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OF

EXPERIMENTAL PATHOLOGY

ON THE REDUCTION OF THE BACTERICIDAL POWER OF BLOOD WHICH IS EFFECTED BY ADDING TO IT CITRATE OF SODA AND OTHER DECALCIFYING AGENTS; AND ON THE QUESTION WHETHER BLOOD SO TREATED SHOULD BE EMPLOYED FOR IMMUNO-TRANSFUSION.

LEONARD COLEBROOK, M.B., B.S.(Lond.),
Assistant, Bacteriological Department, Medical Research Council,

AND

E. J. STORER, M.R.C.S.(Eng.), L.R.C.P.(Lond.),
Assistant, Inoculation Department, St. Mary's Hospital.

From the Inoculation Department, St. Mary's Hospital, London.

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PART I.—ON REDUCTION OF THE BACTERICIDAL POWER OF BLOOD BY DECALCIFYING AGENTS.

Experiment 1.—Blood from the vein of a normal man (A. F. H—) was divided into two parts. One of these was defibrinated by agitation in a test-tube. To the other was added 1/20th of its volume of 5 per cent. sodium citrate. The two samples of blood were then compared with respect to their bactericidal power for staphylococci. This was done by the method of "inculturing" in "slide cells" described by Wright, Colebrook and Storer (1923). Fifty c.mm. volumes of each sample of blood were mixed with 2·5 c.mm. volumes of a graduated series of dilutions of a staphylococcus broth culture, and then transferred to the slide cells, *i.e.* shallow chambers formed between two microscopic slides. After 24 hours' incubation in these cells at 37°C. the number of colonies which had grown in the blood was as shown in Table I.

TABLE I.

	No. of staphylococci implanted in the blood (as determined by an agar count).		
	*(954)	(318)	106
Number of colonies which grew—			
In defibrinated blood	8	3	2
In citrated blood.	100–130	76	20

This experiment was repeated and a similar result obtained on seven occasions, four normal bloods in all being used. The amount of citrate added to the blood was sometimes 0·5 per cent., at other times, as in the above experiment, 0·25 per cent.

Experiment 2.—In this case streptococcus was used as the test organism. By reason of the fact that the variety of streptococcus here in question failed to produce visible colonies in the blood the method of slide cells was discarded. Instead, the method of "implanting and explanting" was employed. Three dilutions of a broth culture of streptococcus (*fæcalis* type, strain "Allen") were prepared, and 2·5 c.mm. volumes of each of these were implanted into a series of 50 c.mm. volumes of (*a*) defibrinated blood (L. C—'s), (*b*) the same blood citrated (0·25 per cent. of citrate). After incubation in pipettes for two hours at 37°C. these infected bloods were blown out into melted agar and the latter poured into Petri dishes. The bactericidal effect achieved by the several samples of blood during the two hours' incubation is shown in Table II.

TABLE II.

	No. of streptococci implanted in the blood (as calculated from agar counts).		
	(1116)	(372)	124
Number of colonies which grew—			
From the defibrinated blood	79	16	15
From the citrated blood	312	91	19

These experiments, and several more carried out with the bloods of other normal individuals, which gave a similar result, show that the hæmobactericidal power exerted upon staphylococcus and streptococcus is considerably impaired by the addition of sodium citrate. So small an addition as 0·25 per cent., which represents almost the minimum that will suffice to prevent the blood clotting, has this effect upon the blood. By similar experiments it was shown that the bactericidal efficiency of the blood is also reduced when coagulation is prevented by other decalcifying agents, *e.g.* by ammonium oxalate and sodium phosphate.

Experiment 3.—The bactericidal power of normal blood (defibrinated) for staphylococcus was compared with that of the same normal blood to which 0·05 per cent. of ammonium oxalate had been added. (Procedure as in Experiment 1 above.)

* The figures shown in brackets in this and subsequent tables represent calculated, not actual counts.

TABLE III.

	No. of staphylococci implanted in the blood (as determined by an agar count).		
	900	300	100
Number of colonies which grew—			
In defibrinated blood	10	6	1
In oxalated blood	150–200	55	28

Experiment 4.—As above, but sodium phosphate (0·4 per cent.) was added to the blood instead of ammonium oxalate.

TABLE IV.

	No. of staphylococci implanted in the blood (as determined by an agar count).
	60
Number of colonies which grew—	
In defibrinated blood	8
In phosphated blood	30

Discussion of the above Results.

The work of Wright, Colebrook and Storer (1923) has made it clear that the killing of staphylococci and streptococci in normal whole blood is brought about chiefly by the agency of leucocytes acting in conjunction with the opsonic power of the serum. The serum exercises little, if any, direct bactericidal effect. We are therefore entitled to infer that the loss of bactericidal power demonstrated in Experiments 1 to 5 was due either to inhibition of the opsonic factor in the serum or to some interference with the functions of the leucocytes.

To enable us to decide between these alternatives the following experiment was carried out:

Experiment 5.—Comparison of the opsonic power of citrated plasma with that of serum. Blood from a normal man (L. C—) was divided into two parts, one being citrated (0·25 per cent. of citrate), the other defibrinated. Equal volumes of the serum and citrated plasma respectively derived from these two samples of blood were mixed with a thick suspension of streptococcus (*fæcalis* type), and incubated for two hours in order to opsonize the cocci. The latter were then washed in two changes of Ringer's solution, and the degree of opsonic influence which had been exerted upon them afterwards determined by mixing one volume of each coccal suspension with two volumes of washed corpuscles, and incubating for 15 minutes. The phagocytic intake was then counted:

Cocci treated with the serum	4·9	per leucocyte.
„ „ „ „ citrated plasma . . .	5·7	„ „
„ which had not been sensitized (spontaneous phagocytosis)	0·3	„ „

A second experiment of the same kind gave the following results:

Cocci treated with the serum	12·8	per leucocyte.
„ „ „ „ citrated plasma . . .	10·3	„ „
„ which had not been sensitized . . .	0·1	„ „

These results indicate that the opsonic power of the plasma is little—if at all—affected by the addition of sodium citrate to the blood; and they point to *the loss of bactericidal power caused by such an addition being attributable to interference with some functions of the leucocytes.*

A further point will be noticed on referring back to the results of Experiments 1 and 2. In Experiment 1 we see that the number of staphylococci which survived in the defibrinated blood when the implantation was 318 (*i.e.* 6360 per c.cm. of blood) was only 3, as against 2 when the implantation was 106 (*i.e.* 2120 per c.cm. of blood). Similarly, in Experiment 2 the number of staphylococci which survived in the defibrinated blood when the implantation was 372 (*i.e.* 7440 per c.cm. of blood) was only 16, as against 15 when the implantation was 124 (*i.e.* 2480 per c.cm. of blood). In the paper previously referred to, Wright, Colebrook and Storer have pointed out that numerical relationships of this kind, viz. 3 to 2, or 16 to 15, where one would expect a ratio of 3 to 1, strongly suggest that an immunizing response has taken place in the bloods with the higher implantation.

The point we now wish to call attention to is that in these same experiments *where the staphylococcal implantations were made into citrated blood, instead of defibrinated blood, there is no such evidence of an immunizing response.*

The question now arises—Why does the addition of decalcifying agents to blood have so marked an effect upon its leucocytes? We do not know.

The explanation which first occurs to the mind is that these drugs exercise a directly toxic effect, *per se*, upon the leucocytes. The following experiment suggests, however, that this is not the case:

Experiment 6.—Here again blood from a normal man (A. F. H—) was divided into two parts, one being citrated (0·25 per cent. of citrate), and the other defibrinated. The defibrinated portion was then itself divided into two parts, one receiving an addition of sodium citrate comparable to that added to the fresh blood. The bactericidal efficiency of the three samples was then tested by the method of slide cells, staphylococcus being employed as the test organism. The result is shown in Table V.

TABLE V.

	No. of staphylococci implanted in the blood (as determined by an agar count).		
	954	318	106
Number of colonies which grew—			
In defibrinated blood	8	3	2
In citrated fresh blood	130	76	20
In defibrinated blood to which citrate was added	21	10	2

This experiment shows that sodium citrate acting in plasma has, for some reason as yet unexplained, a much greater effect upon the bactericidal functions of the leucocytes than it has when added to defibrinated blood.

PART II.—ON THE QUESTION WHETHER BLOOD IN WHICH COAGULATION HAS BEEN PREVENTED BY MEANS OF DECALCIFYING AGENTS SHOULD BE EMPLOYED FOR THE PURPOSE OF IMMUNO-TRANSFUSION.

Here a word of introduction is perhaps necessary. Sir Almroth Wright (1919) has advocated the treatment by immuno-transfusion of heavily-infected patients who, by reason of the poisoning of their leucocytes, are unable to make an adequate immunizing ("epiphylactic") response. In a recent paper dealing with this procedure the present authors (1923) have explained that immuno-transfusion seeks to bring aid to such patients in two ways: firstly by directly enriching their blood-fluids in respect of protective substances, thus conferring upon them a temporary *passive immunity*; and, secondly, by the introduction of a large body of *fresh leucocytes* capable of making an immunizing response, *i. e.* of furnishing protective substances.

The experiments which follow were designed to show whether the addition of a decalcifying agent to a donor's immunized blood was in any degree inimical to the aims of immuno-transfusion as here set out. We required to know (*a*) whether the addition of the decalcifying agent diminished the bactericidal power of the blood fluids of the donor who has made immunizing response to an inoculation of vaccine, and (*b*) whether such an addition had the same detrimental effect on the bactericidal efficiency of his leucocytes as has been demonstrated in the case of those of the normal blood.

Experiment 7.—Here comparison was made between the bactericidal power to staphylococcus of (1) the serum of a normal man's blood, (2) the serum of the same man's blood taken after he had made an immunizing response to a dose of vaccine, (3) the citrated (0·25 per cent. citrate) plasma prepared from the same blood.* The bactericidal power of the samples of serum and plasma respectively, derived from these bloods, was determined by the "inculturing method," 50 c.mm. volumes being incubated in slide cells after implantation with 2·5 c.mm. volumes of a diluted culture of staphylococcus.

TABLE VI.

	No. of staphylococci implanted (as shown by an agar count). 100
Number of colonies which grew—	
In serum of blood taken before inoculation . . .	69
In serum of blood taken 4 hours after inoculation . .	42
In citrated plasma of blood taken 4 hours after inoculation	34

This experiment shows (1) that, as a result of the inoculation of vaccine, the serum acquired an enhanced power of directly killing staphylococci, this increase of power being at the rate of 27 cocci per 50 c.mm. of serum, or 540 cocci per c.cm., and (2) the addition of citrate to the blood thus immunized did not deprive it of any of its increased sero-bactericidal power.

* Since the experiments of Part I suggest that all decalcifying agents affect the blood in a similar manner with respect to its bactericidal efficiency, and sodium citrate has been most generally employed for the purpose of therapeutic transfusions, this salt has been used exclusively in Experiments 7 to 9.

Experiment 8.—This experiment was similar to that just described, but the determinations of bactericidal power were made upon the whole blood, with and without citrate, instead of upon the serum and citrated plasma. The details of the immunization and the testing of the bloods were as stated under Experiment 7. The result is shown below:

TABLE VII.

	No. of staphylococci implanted in the blood (as shown by an agar count).		
	1000	333	111
Number of colonies which grew—			
In defibrinated blood taken before inoculation .	70	27	9
In defibrinated blood taken 4 hours after inoculation	33	6	2
In citrated portion of same blood (viz. 4 hours after inoculation)	120	55	19

Here we see that (1) the capacity of the whole blood to kill staphylococcus was considerably increased as a result of the inoculation of vaccine, and that (2) the addition of citrate to the blood thus immunized not only deprived it of its increased bactericidal power, but also reduced that power to a decidedly lower level than was found before the immunization. Evidently the leucocytes of the immunized blood are affected by the addition of citrate just as those of normal blood.

We conclude from these experiments that the employment of citrated blood for immuno-transfusion, although it is compatible with the programme of conferring a temporary passive immunity upon the patient, is not well calculated to serve the additional purpose of conveying to him a large number of normal, active leucocytes.

It may be suggested, however, that although the experiments described point to interference with the functions of the leucocytes when these cells are retained in citrated plasma for the whole duration of the experiment, *i.e.* 24 hours, recovery of their normal functioning might quickly occur when citrated blood came to be diluted, after a relatively short time, with a large volume of uncitrated blood.

The following experiment was carried out with this possibility in view:

Experiment 9.—Here the leucocytes of citrated blood were removed from their unfavourable environment and restored to a congenial one. Bactericidal tests were then carried out to determine how far they had recovered their functional activity. One part of a sample of blood obtained from a normal man (L. C—) was citrated (0·5 per cent. citrate), the other defibrinated. After standing on the bench for two hours the two samples were centrifuged and their corpuscles twice washed with Ringer's solution. To these washed corpuscles was then added an equal volume of serum previously derived from the defibrinated blood, and the reconstituted bloods thus obtained were allowed to stand on the bench for a further period of one hour. Their bactericidal

efficiency towards staphylococcus was then tested by the method of slide cells. The result was as follows:

TABLE VIII.

	No. of staphylococci implanted in the blood (as shown by an agar count).			
	848	424	212	106
Number of colonies which grew—				
In blood of which the corpuscles had previously been exposed to citrate	98	51	38	15
In control blood	75	30	15	8

From this result it may be inferred that the leucocytes derived from citrated blood-plasma did not recover their bactericidal efficiency within the period of the experiment.

Employment of Citrated Blood for Simple Transfusion.

As an alternative to citrated blood for immuno-transfusion, the authors (1923) have described a method of transfusion of defibrinated blood which has proved quite as easy to carry out as the transfusion of citrated blood.

When simple transfusion is performed with a view to making good a deficiency of red blood-corpuscles in the circulation, whether in cases of traumatic hæmorrhage, of primary anæmia or of shock, one can perhaps afford to neglect the reduction of bactericidal efficiency which results from adding citrate to the donor's blood. There are, however, other considerations which make us question the advisability of using blood so treated even in cases of this kind. Experience has taught that transfusions of citrated blood are very frequently followed by constitutional "reactions" of greater or less severity, and that these are not eliminated by scrupulous care in the choosing of donors whose blood is "compatible" with that of the recipient patients.

Unger (1917) has stated that febrile "reactions" occur after 60 per cent. of transfusions of citrated blood, and "chills" after 57 per cent. In Lederer's (1923) experience of 49 cases the reactions occurred in 49·5 per cent. The nature and causation of these reactions is still obscure, but it seems clear that citrating the blood favours their occurrence. Unger observed only 14 reactions in connection with a series of 165 transfusions of unaltered blood, while Lederer reports that none occurred after 40 transfusions of this kind carried out by him. Similarly, in our hands, the injection of defibrinated blood has very seldom given rise to any disturbance—not more than twice in the last 45 cases.

SUMMARY AND CONCLUSIONS.

Experiments are described which show that the addition of decalcifying agents to normal blood considerably reduces its power to kill staphylococcus and streptococcus.

The result is attributable to interference with some of the functions of the leucocytes.

Blood which has an enhanced bactericidal power to staphylococcus and streptococcus, resulting from a previous inoculation of vaccine, is affected by the addition of sodium citrate in the same way as normal blood. In consequence of this it is recommended that citrated blood should not be employed for "immuno-transfusion."

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THE EFFECT OF RADIATION ON THE BACTERICIDAL POWER OF THE BLOOD.

L. COLEBROOK, A. EIDINOW, AND LEONARD HILL.

From the National Institute for Medical Research, London, and the Inoculation Department, St. Mary's Hospital, London.

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THE influence of ultra-violet radiation in preventing or curing rickety changes in bone of animals or children fed on a deficient and rickets-producing diet has been established by Huldschinsky (1920), Hess (1921, 1922), etc., and confirmed, among others, in the case of young rats by T. A. Webster and Leonard Hill (1923). It has also been shown that young rats fed on a diet deficient in vitamin A suffer less from infection of the eyes and ears, and compared with those kept in the dark, live much longer when freely exposed to sun and open air, during continued fine summer weather (Powers, Park and Symond, 1923). Likewise chicks when fed on a rickets-producing diet grow up not only free from rickets, but much larger and stronger when given unrestricted exposure to sunshine and open air in place of confinement indoors (Hart, Steenbeck, Lepkovsky and Halpin, 1923). It has been recently shown, too, that lupus of a kind incurable by local Finsen light treatment may be cured by arc-light baths applied to the whole body (Sequeira, 1923).

The experience of O. Bernhard (1899), Rollier (1905, 1923), Gauvain (1920), etc., in the treatment of surgical tuberculosis has demonstrated the beneficial effect of exposure of the body to open air and sunlight. Gauvain finds that the curative effect proceeds much better in England in periods of fine summer weather. The Alps, compared with England, offer the advantage

of far more hours of sunlight and a sun richer in ultra-violet rays. There O. Bernhard has successfully exposed war wounds to sun and open-air treatment; there, too, Rollier builds up the health of delicate children by exposure of their naked bodies to sun and open air not only in summer but in the snowfields in winter—not only in games, but when sitting at desks and having lessons. The effect on health of open-air schools wherever tried, however, has proved most favourable. Now it has been shown by Argyll Campbell and Leonard Hill (1922, 1923) that the resting metabolism of the body is put up 50 to 100 per cent. in children and adults exposed to open-air treatment. The high cooling power of open air has a curative effect in stimulating muscular tone, appetite and digestion by preventing constipation and promoting activity of the circulation and deeper breathing. Leonard Hill has pointed out that the breathing of cool air, holding as it does but little moisture even when saturated, has a beneficial effect on the respiratory membrane by enhancing the flow of blood and lymph through, and the outflow of secretion from it, to make good evaporation and loss of heat from the surface—all this resulting in a cleansing and immunizing effect.

At the same time radiation acts on the skin. The dark heat rays are absorbed by water and penetrating to, and being absorbed by, the surface layers of wet tissue, warm these.

The visible rays, on the other hand, penetrate to the cutaneous and even deeper blood-vessels, and are absorbed by the blood, and warm it. According to Sonne (1921), this local heating of the blood is of importance in enhancing the immunizing processes which the skin carries on. The subcutaneous tissues may be heated even to 47½°C. when the surface of the skin is radiated with visible rays up to the point of endurance (confirmed by Argyll Campbell and Leonard Hill). Sonne finds that such radiation with visible rays increases the resistance of guinea-pigs to a minimal lethal dose of diphtheria toxin, but the evidence of this adduced by him is by no means strong. T. Hansen (1921) claims that after injection of typhoid vaccine, the agglutinating power of the blood for typhoid bacilli is increased by subsequent ultra-violet radiation of the body.

There are no ultra-violet rays present in the high sunlight on clear days shorter than 2900 A.U. It is the rays about 3000 A.U. which appear to have the power to produce sun-burn, *e.g.* erythema, desquamation, pigmentation (Hausser and Vahle, 1921); the longer ultra-violet rays and visible rays of sufficient energy probably share in this power. Shorter rays have great power to kill unicellular organisms, a power increasing as the rays shorten, but their power to penetrate the living substance falls off very rapidly as they become shorter, until even one microbe suffices to shade another.

It is clear, then, that these rays do not act by destroying infecting microbes in wounds and tubular lesions, but by putting up the immunizing properties of the body as a whole. The difference in penetration of rays from a carbon tungsten arc lamp and mercury vapour lamp is shown by the following: When an arc light is concentrated on the frog's tongue and this is kept cool by irrigation, stasis in the capillaries shortly results (Dreyer and Jansen, 1905). This we suggest is through the action of visible and longer ultra-violet rays, for we find stasis is not produced by exposure to the mercury vapour lamp, the

radiation from which is rich in shorter ultra-violet rays which do not penetrate to the blood-vessels. This lamp does, after a time, however, produce stasis in the capillaries of the frog's mesentery because this membrane is thin enough to allow some penetration of harmful rays. As is well known after exposure of the skin to the mercury vapour lamp there follows some two hours later erythema and subsequently desquamation and pigmentation. It appears probable then that the rays of this lamp act on the deeper growing cells of the epidermis, and by damaging these and irritating the superficial nerve plexus, set up a secondary dilatation of the blood-vessels.

The carbon arc, on the other hand, is rich in rays (heat, visible, etc.), which penetrate and cause flushing of the skin during exposure. Some writers (Rollier, Gauvain) suggest that pigment acts as a transformer converting certain rays into others which have a biological action and a beneficial effect. We know no more than that pigment acts as a screen and protects the deeper structures from excessive radiation, and converts visible radiation into dark heat, which by stimulating the nerve plexus in the skin provokes sweating. The sweat moistening the skin, by reflecting light and evaporating off heat, in its turn protects the body against excess of radiation.

In this paper we shall describe results which appear to show that any insult to the skin, whether produced by ultra-violet and visible rays, or by dark heat, or by blistering agents, appears to provoke or increase the defensive power of the body.

The absorption into the blood of the products of tissue damage, or the enhanced absorption of normal tissue products produced by such damage and the consequent erythema and œdema, appears to be a natural excitant of immunizing mechanisms. As the test of these, we have studied the bactericidal power of the whole blood.

EXPERIMENTAL.

General plan of the experiments.—(1) The back or abdomen of rabbits after removal of fur was exposed to various radiations, *e.g.* ultra-violet, luminous, or infra-red rays. (2) While warming the ear so as to dilate the vessels, samples of blood were taken from a vein before, and at stated intervals after, the period of irradiation, and these samples were then tested with regard to their power to kill staphylococci. (3) In several cases the bactericidal power of the serum as well as that of the whole blood was determined.

Details of procedure.—Each sample of blood was defibrinated by whipping with a sterile slender glass rod bent in the form of a U.

The bactericidal power of the several samples was determined by the method of "inculturing" in "slide cells" recently described by Wright, Colebrook and Storer (1923). For this purpose several 50 c.mm. volumes of blood were mixed with 2·5 c.mm. volumes of a progressive series of dilutions of a young broth culture of staphylococcus, and the mixtures transferred to shallow chambers formed between two microscopic slides (slide cells) for incubation overnight at 37°C. The cocci which survive the bactericidal effect of the blood grew into colonies visible to the naked eye and were readily counted. Comparison of the numbers of colonies so obtained with that of the staphylococci originally implanted in the blood this figure is arrived at by

making precisely the same implantation into melted agar at 48 C. and pouring the latter into a Petri dish) gave an estimate of the bactericidal effect exerted by the particular sample of blood.

When the bactericidal power of the serum rather than that of the whole blood was under investigation similar mixtures were made of the serum to be tested and of a diluted broth culture of staphylococcus, and these mixtures were incubated either in slide cells, as in the case of the blood tests, or in long capillary pipettes. By restricting the implantation of microbes to a number between 20 and 80 for each 50 c.mm. sample of serum tested, we ensured the growth of discrete colonies which could be easily enumerated. By comparison of the number so obtained with that of the cocci implanted—as shown by a separate agar count—we determined the bactericidal effect, if any, achieved by the particular serum.

Two considerations made it advisable to employ a range of dilutions of the microbic culture rather than one single dilution on all occasions:

(1) The number of living organisms in the culture will vary somewhat from day to day.

(2) The bactericidal effect achieved by a given sample of blood has been shown by Wright, Colebrook and Storer to depend in some measure upon the number of organisms used in testing it. If we implant more than a certain number of staphylococci into the blood the percentage killed will begin to fall away, and with some blood at any rate a *smaller* implantation than the optimum will also give us a reduced bactericidal effect. Presumably in this latter case the infection is insufficient to evoke an immunizing response on the part of the white blood-corpuscles.

By employing a range of 3 or 4 suitable dilutions of our microbic culture, we aimed at registering in one of them approximately the maximum bactericidal effect.

A typical experiment.—The following is the result of an experiment conducted on a rabbit, the back of which was shaved and exposed for half an hour to the mercury vapour lamp (M.V.L.), enough to produce a subsequent erythema and desquamation of the skin:

TABLE I.

Number of colonies which grew—	Number of staphylococci implanted (as shown by agar count): (120.)*	(40.)*	13.
In blood taken before animal was exposed to light	70	24	14
In blood taken ½ hour after exposure	2	1	0
In blood taken 2 hours after exposure	24	4	2
In blood taken 3 hours after exposure	38	9	4

In Table II (p. 58) we have summarized the results obtained in all the experiments in which the skin of rabbits, piglings and man was exposed to radiation from one or another luminous source.

* Figures in brackets are calculated, not actual counts.

The bactericidal effects achieved by the bloods taken before and after irradiation are here expressed as percentages of staphylococci killed. An average was taken of the percentage killed with each implantation of staphylococci. Observations on some control animals treated in a similar fashion, but not exposed to the artificial light, are included.

TABLE II.

EXPERIMENT	EXPOSURE TO	DISTANCE	TIME OF EXPOSURE MINUTES	% KILLED BEFORE EXPOSURE	MAXIMUM % KILLED 1½-3 HRS AFTER EXPOSURE	DIFFERENCE	RISE OF KILLING POWER %	
CONTROL RABBIT SHAVED AND FASTENED DOWN	NO EXPOSURE		—	78	79	—	—	
CONTROL SHAVED AND LAPAROTOMY PERFORMED. RABBIT H.	"		—	36	37	—	—	FIG. 1
RABBIT A	M. V. L	9 INCHES	30	57	85	28	49	
" B	"	"	"	40	99	59	147	FIG. 2
" C	"	"	"	63	91	28	44	
" F	"	"	"	62	99	37	59	
" AA	"	"	"	35	100	65	185	
" —	M.V.L. WITH PHOTO PLATE GLASS SCREEN	"	"	67	82	15	22	
" O	M.V.L. CADMIUM SPARK	"	"	54	99	45	83	FIG. 3
" S	SUN - OCTOBER MID-DAY	—	120	67	99	32	49	FIG. 4
" X	CARBON ARC	18 "	30	62	94	32	51	FIG 5
" K	ARC WITH GLASS AND WATER FILTER.	DIFFUSELY FOCUSSED	30	77	99	22	28	FIG. 6
" M	ARC WITH FERROUS AMMONIUM SULPHATE SOL. FILTER.	"	"	74	78	4	5	FIG. 7
" N	ARC WITH IODINE IN POTASS IODIDE SOL. FILTER	"	"	65	93	28	43	FIG. 8
PIGLING A SHAVED BACK	M. V. L	18 INCHES	"	66	93	27	41	FIG. 9
" B "	"	"	"	84	96	12	14	
MAN L.C. BACK AND FRONT OF TRUNK	"	"	40	86	97	11	14	FIG. 10
" N. " "	"	"	30	60	92	32	53	
" A.W. " "	"	"	"	77	90	13	17	

EXPERIMENT	EXPOSURE TO	TEMPERATURE OF EXPOSED SKIN	DISTANCE	TIME OF EXPOSURE MINUTES	% KILLED BEFORE EXPOSURE	MAXIMUM % KILLED 1½-2 HRS AFTER EXPOSURE	DIFFERENCE	RISE OF KILLING POWER %	
RABBIT P	HEAT, ELECTRIC STOVE	44-47°C		30	58	99	43	74	FIG. 11
" —	" " "	40°C		"	70	96	26	37	
" U	" " "	40°C		"	89	91	2	2	FIG. 12
" W	HOT WATER BATH 40-44°C			"	60	92	32	53	FIG. 13
" —	DARK HEAT	42°C		"	79	81	2	3	
" G	ILEUM M.V.L.		7 INCHES	15	30	88	58	196	FIG. 14
" 54	MUSTARD LEAF ON SHAVED BELLY			60	41	89	48	117	FIG. 15
" —	MUSTARD & LINSEED POULTICE ON SHAVED BELLY. REPEATED ONCE			60 EACH	67	78	11	16	

On one occasion streptococcus (*fæcalis* type) instead of staphylococcus was used as the test microbe. Since this organism does not grow into visible colonies when implanted in undiluted blood in slide cells the technique employed in the previous experiments had to be modified as follows:

The specimens of blood taken from the rabbit before and after irradiation with ultra-violet light were implanted with a diluted broth culture of streptococcus (in the proportion of 20 to 1), and were then incubated at 37° C. for 2 hours to enable the bactericidal effect of

the bloods to be exerted. At the end of this period of incubation a 50 c.mm. sample was taken from each, mixed with melted agar at 48° C., and the latter poured into a Petri dish. The result is shown in Table III:

TABLE III.

Number of colonies which grew—	Number of streptococci implanted in each sample of blood (as determined by an agar count), 168.	Streptococci killed. %
From blood taken before exposure of animal to Hg. vapour lamp	89	47
From blood taken ½ hour after exposure	69	61
From blood taken 1 hour after exposure	45	73

The increased bactericidal effect here shown was at the rate of 880 cocci per c.cm. of blood.

The mercury vapour lamp used for irradiation was Kelvin, Bottomley and Baird's, taking two ampères at 200 volts. At 9 in. distance infusoria in a shallow quartz cell are killed by this lamp in about 10 minutes, and exposure for this time of the upper inner surface of the arm results in well-marked erythema. The carbon arc was one taking 10 ampères at 200 volts used naked at a distance of 18 inches. It was diffusely focussed on the shaved area by means of a metal concave reflector when filters were used.

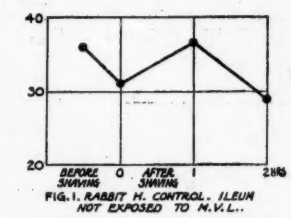

FIG. 1. RABBIT H. CONTROL. ILEUM NOT EXPOSED TO M.V.L..

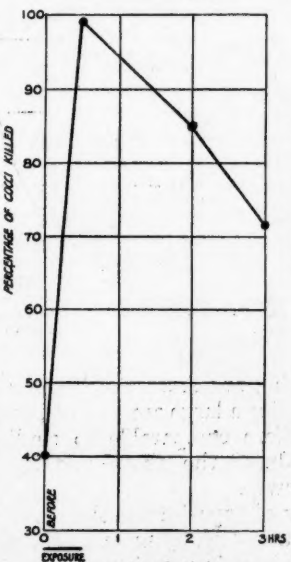

FIG. 2. RABBIT B SHAVED BACK EXPOSED TO M.V.L. FOR ½ HOUR.

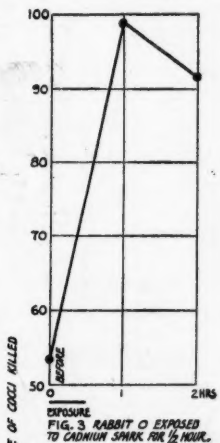

FIG. 3 RABBIT O EXPOSED TO CADMIUM SPARK FOR ½ HOUR.

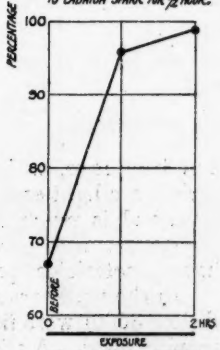

FIG. 4. RABBIT S EXPOSED TO SUN FOR 2 HOURS

In the first series of experiments an area of skin of the back or abdomen of the animal 6 in. by 4 in. was exposed to the M.V.L. for half an hour. A series of investigations of the bactericidal power of the blood showed that such irradiation resulted in an increased killing of staphylococci and streptococci both in the blood and serum. After irradiation the skin showed a characteristic erythema and œdema; a few days later desquamation and slight pigmentation were visible. A series of control experiments

showed that this effect was not due to shaving of the hair. As in some cases a slight rise in hæmobactericidal power was observed after barium sulphide had been employed as depilator, and in order to avoid the possibility of any photo-sensitization effects of this substance, the animals were shaved with soap and a razor and were not irradiated until the day following.

The M.V.L. is extremely rich in short ultra-violet rays, but does not emit much heat. The lamp used by us only gave a rise of 1–2°C. in skin temperature after irradiation at 9 in. distance for half an hour. This lamp emits visible

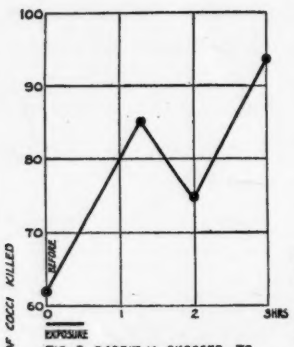

FIG. 5 RABBIT X. EXPOSED TO NAKED CARBON ARC FOR ½ HOUR.

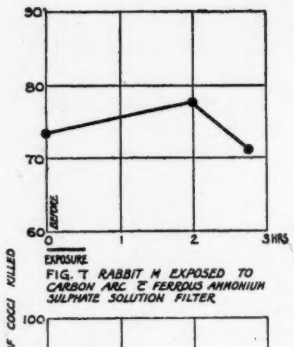

FIG. 7 RABBIT M EXPOSED TO CARBON ARC & FERROUS AMMONIUM SULPHATE SOLUTION FILTER.

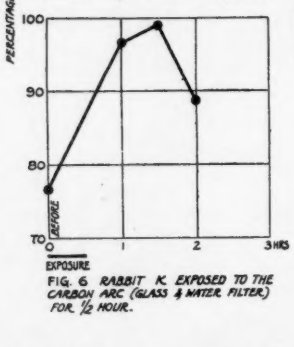

FIG. 6 RABBIT K. EXPOSED TO THE CARBON ARC (GLASS & WATER FILTER) FOR ½ HOUR.

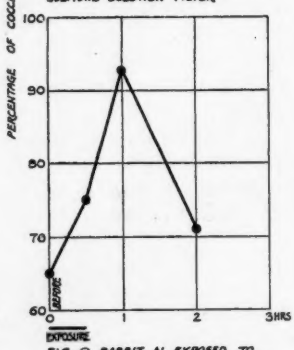

FIG. 8 RABBIT N EXPOSED TO CARBON ARC & IODINE IN POTASS. IODIDE SOLUTION. FILTER. (HEAT RAYS)

and heat rays, which come from the heating of the quartz container. The carbon arc light contains a large amount of heat, visible and longer ultra-violet rays, and is much more comparable to sunlight. Exposure to the naked carbon arc also produced the above reaction in the skin and put up the hæmo-bactericidal power.

In the next series of experiments the short ultra-violet rays of the M.V.L. (below 3000) were filtered off by means of a plain glass screen, viz. a photographic plate. A slight skin reaction was obtained and the hæmo-bactericidal power increased. A similar effect resulted on condensing the arc light through a glass and water screen to take out the heat and shorter ultra-violet rays.

When the heat and ultra-violet rays were filtered off by using a solution of ferrous ammonium sulphate as filter, and the animal was irradiated with luminous rays only for half an hour, there resulted no significant skin reaction and no rise in hæmo-bactericidal power; conversely, when using a filter of iodine in potassium iodide solution, an animal was irradiated with red luminous and heat rays, a skin reaction developed and also a rise in hæmo-bactericidal power.

Further experiments in which the animal was exposed either to an electric heater (red and dark heat rays) or to dark heat by radiation from a metal plate placed in front of a gas stove showed that heat rays when associated with skin reactions give a rise in hæmo-bactericidal power. In experiments in which the animal was not shaved and in which the temperature was completely regulated and no skin reaction occurred, there was no rise in this power.

In an experiment in which pure ultra-violet rays from a cadmium spark were employed, there followed reaction in the skin and a rise in hæmo-bactericidal power. Exposure to the sun also gave a positive result.

The reaction is not specialized to skin tissues—as exposure of a coil of the ileum for 15 minutes to the M.V.L. no less gave a positive result. A control animal, in which laparotomy was performed but the gut not exposed to light, gave no result.

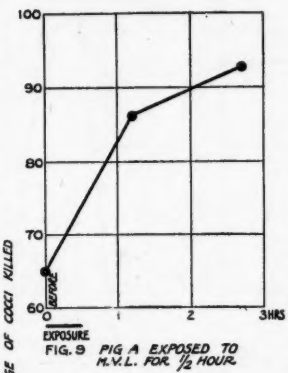

FIG. 9 PIG A EXPOSED TO M.V.L. FOR ½ HOUR.

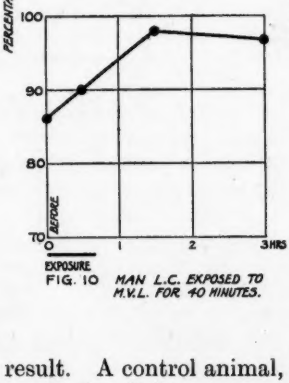

FIG. 10 MAN L.C. EXPOSED TO M.V.L. FOR 40 MINUTES.

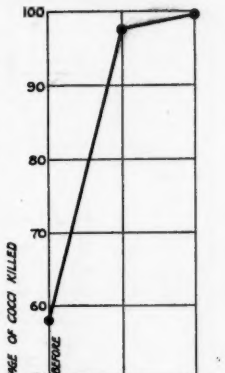

FIG. 11 RABBIT P EXPOSED TO ELECTRIC HEATER FOR ½ HOUR

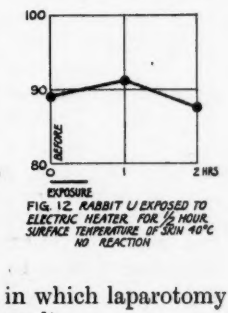

FIG. 12. RABBIT U EXPOSED TO ELECTRIC HEATER FOR ½ HOUR. SURFACE TEMPERATURE OF SKIN 40°C NO REACTION

Irradiation of defibrinated blood in a quartz flask for varying periods of 1 to 20 minutes did not give any change in hæmo-bactericidal power of this blood.* The reaction appears therefore to be due to the irradiation of tissues and circulating of blood producing changes in these which excite an increase in the immunization quality of the blood of the animal.

Exposure of piglings and man to the M.V.L. has given similar results.

* We have some evidence that this blood when put back into the animal increases the hæmo-bactericidal power, and that it is the irradiation of the corpuscles and not of the serum which is effective.

An application of mustard leaf or mustard poultices to the shaved skin of rabbits which produces erythema and œdema also raises the hæmo-bactericidal power.

As the figures given in the table are mean percentage averages of the bactericidal power of the blood, they do not fully express the true position in these cases when this power is initially high. The marked change after

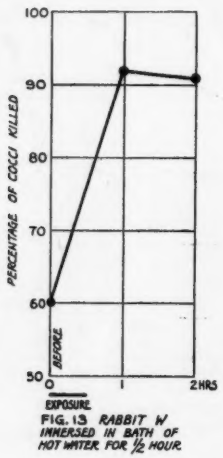

FIG. 13. RABBIT W IMMERSED IN BATH OF HOT WATER FOR ½ HOUR.

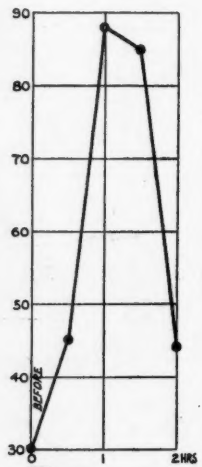

FIG. 14. RABBIT G ILEUM EXPOSED TO H.V.L. FOR ¼ HOUR.

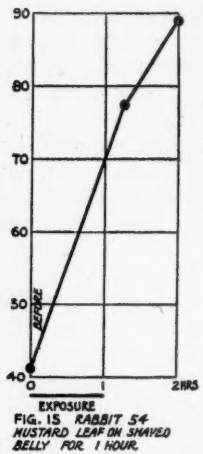

FIG. 15. RABBIT 54 MUSTARD LEAF ON SHAVED BELLY FOR 1 HOUR.

irradiation in the number of colonies is very striking when the slide cells are inspected. The graphs show the temporary nature of the change.

ANALYSIS OF THE CHANGE IN IRRADIATED RABBIT'S BLOOD.

Bactericidal tests were made to determine whether (*a*) the serum or (*b*) the white blood-corpuscles or (*c*) both of these elements had acquired an increased power to kill staphylococci as a consequence of the exposure to light, etc.

Serum.

The method was that described above.

The normal rabbit's blood-serum may be said to have little or no bactericidal action on staphylococcus.

After the animal had been irradiated, the serum frequently, but not always, showed an undoubted bactericidal action. Generally speaking, if the whole blood in the slide cells showed a positive result, we got a positive result also in the serum, but the latter was manifested a little later than the result in the blood, *e. g.* blood might show a maximal effect after 1 hour and serum not until 2 hours.

The following are illustrative experiments:

Number of colonies which grew—	No. of cocci implanted in the serum, 44.	Killing power of the corresponding blood (tested in slide cells), %.
(1) In serum of blood from rabbit (F) before it was exposed to Hg. vapour lamp	34	65
In serum 2 hours after exposure.	1	99
	No. implanted, 49.	
(2) In the serum of blood from rabbit (K) before exposure to carbon arc-lamp (light screened through water and glass)	44	75
In serum ¾ hour after exposure	44	98
In serum 1½ hours after exposure	0	99·5
	No. implanted, 58.	
(3) In serum of rabbit (S) taken before exposure to sunlight for 2¼ hours	45	66
In serum 1 hour after exposure	29	96·5

White Blood-corpuscles.

Experiments were done to determine whether the bactericidal functioning of the leucocytes proceeds on a higher level after the animal had been exposed to ultra-violet light.

The blood specimens taken from the rabbit before irradiation (A), one hour after irradiation (B), two hours after irradiation (C), were centrifuged, and their sera pipetted off. The following mixtures were then made:

(1)	One vol. of corpuscles derived from blood	A	+	One vol. of serum derived from blood	A.
(2)	do.	B	+	do.	A.
(3)	do.	C	+	do.	A.

The bactericidal efficiency of these three reconstituted bloods—in which, as will be seen, the corpuscle element is the only variable factor—was then tested in the usual way by the method of slide cells.

The result was as shown in the two following experiments:

Number of colonies which grew in—	Number of staphylococci implanted in the blood (determined by an agar count). (120).*	(60).	30.	Staphylococci killed. %
I. Mixture (1)	40	16	11	68
Mixture (2)	16	1	1	91
Mixture (3)	11	4	0	93
II. Mixture (1)	35	4	—	78
Mixture (2)	0	0	—	100
Mixture (3)	0	0	—	100

* The numbers shown in brackets represent calculated, not actual counts.

From these two experiments it appears that the bactericidal efficiency of the corpuscles is enhanced after an exposure to ultra-violet radiations. And in view of Almroth Wright's work, which has demonstrated that most of the bactericidal effect of blood upon serophytic microbes like staphylococcus is referable to the leucocytes, we may legitimately conclude that it is these latter cells which are stimulated to function more vigorously in the blood of irradiated animals.

Antitoxic Content of Serum.

Dr. P. Hartley has found that the irradiation by us of guinea-pigs immunized against diphtheria toxin is without effect on the antitoxic content of the serum.

CONCLUSION.

Both the serum and the leucocytes contribute to an increased bactericidal effect achieved by the blood of rabbits after they have been exposed to various radiations which produce inflammation of the skin. The increase in hæmo-bactericidal power is found also in pigs, and to a less degree in man. It is suggested that the products of the damaged tissue cells evoke this reaction.

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INOCULATION AND IMPLANTATION EXPERIMENTS IN MONKEYS WITH GLANDS FROM CASES OF HODGKIN'S DISEASE.

M. J. STEWART AND J. F. DOBSON.

From the Department of Pathology and Bacteriology, University of Leeds.

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THE experimental results so far obtained in Hodgkin's disease fall into one or other of the three following categories: (1) The production of a tuberculous lesion in experimental animals by the injection of Hodgkin gland emulsions (Cignozzi, 1906; Schaeffer, 1914). (2) The reproduction of the histological changes of the disease as seen in man by the injection of cultures of a diphtheroid bacillus recovered from the lymph-glands in cases of Hodgkin's disease (Bunting and Yates, 1913, 1914). (3) Negative findings (Longcope, 1908; Torrey, 1916; Cunningham and McAlpin, 1923).

These various results are in keeping with the different views as to the ætiology of Hodgkin's disease already arrived at by various observers on other grounds, viz. that it is (1) an atypical form of tuberculosis; (2) a specific infection by a diphtheroid bacillus; (3) a granuloma of unknown ætiology; (4) a neoplastic disease.

It is not our purpose to discuss here the evidence for and against the tuberculous theory of Hodgkin's disease, nor to do more than make passing reference to the diphtheroid theory, but merely to report briefly certain experimental results which we have obtained in monkeys.

At least four groups of observers have already published the results of experiments in these animals. Bunting and Yates (1913, 1914) worked with diphtheroid bacilli which they had recovered from the glands in cases of Hodgkin's disease, and which they administered by subcutaneous injection. They experimented on five *Macacus rhesus* monkeys, and obtained in all of them changes which, in their opinion, resembled those of Hodgkin's disease. In the second monkey of their series they implanted, into the axilla, glands removed from the first monkey, and which had become enlarged as a result of repeated injections of the diphtheroid organism. This second monkey gradually declined, and died at the end of ten weeks; none of the others succumbed. In all five the enlarged glands are stated to have shown the early histological changes of Hodgkin's disease, a pronounced endothelial cell reaction, fibrosis and eosinophil cell infiltration, but in several there was extensive necrosis in addition. These results have never been confirmed. Torrey (1916), who carried out a similar series of experiments in four *rhesus* monkeys, obtained

negative results, and while some observers (Billings and Rosenow, 1913; Bunting and Yates, 1915; Hatcher and Lemmon, 1915) claim to have had good results with vaccine treatment, there is strong evidence against the view that the diphtheroid bacillus in question is the causative agent in Hodgkin's disease (Bloomfield, 1915; Harris and Wade, 1915; Olitsky, 1915; Cunningham, 1917).

Longcope (1908) experimented on two monkeys with glands from three separate cases of Hodgkin's disease. In both animals the subcutaneous injection of a single large dose of lymph-gland emulsion was followed after a few weeks by moderate generalized lymph-gland enlargement, which persisted for about two months and thereafter slowly subsided. A gland which had been excised during the period of enlargement showed simple hyperplasia of the lymphoid follicles and stuffing of the sinuses with cells. Longcope regarded his results as negative from the point of view of Hodgkin's disease.

Cunningham and McAlpin (1923) experimented on two *Macacus rhesus* monkeys and two chimpanzees. The former were under observation for periods of five and six months respectively from the time of operation, when they had had slices of Hodgkin gland implanted retroperitoneally. There was no evidence of Hodgkin's disease *post-mortem*. In the case of the chimpanzees the Hodgkin tissue was implanted into the deep cervical fascia. The animals were kept under observation for two years and were then sent to a circus. One died a year later, apparently of tubercle, the other within a month, and extensive tuberculosis was found *post-mortem*.

PERSONAL OBSERVATIONS.

Our own experiments were carried out on three *Macacus rhesus* monkey, and one bonnet monkey with material taken from two cases of Hodgkin's disease, the diagnosis having been made by microscopic examination of excised glands. In both the glands were the seat of typical, active cellular Hodgkin's disease, but whereas in Case 1 there was a well-marked eosinophilia, in Case 2 eosinophil cells were absent. Case 1 was used for all three *rhesus* monkeys, Case 2 for the bonnet monkey only. For the purpose of excluding tubercles guinea-pigs were inoculated at the time of the original diagnostic examinations, with negative results. In each experiment laparotomy was performed, and some small chips of a Hodgkin gland just excised from the patient were implanted into some of the mesenteric lymph-glands, usually those nearest the ileocolic angle, or into the spleen, or both.

Experiment 1.—Female *Macacus rhesus*. The abdomen was opened, and a number of Hodgkin chips were inserted into two mesenteric glands situated close to the ileocolic angle, the peritoneum being first incised, and then reunited by a suture. The animal made a good recovery and remained well. Nineteen weeks later it was accidentally killed with chloroform while being anæsthetized for a second experiment. *Post-mortem* a number of mesenteric lymph-glands were found to be enlarged. One, from the seat of implantation, contained an abscess cavity lined by a curious palisade formation of large multinucleated giant-cells (Plate). Elsewhere in the same gland there were one or two small solid nodules consisting of a mass of similar giant-cells,

with intermingled lymphocytes, plasma-cells and neutrophil polymorphs. The giant-cells were of the foreign body type, with abundant foamy cytoplasm, which was sometimes of a curious brown colour in hæmalum and eosin sections, and nuclei which were often peripherally arranged. No tubercle bacilli nor other organisms were seen in sections. Other glands, away from the site of implantation, were also examined. They showed unusually large lymphoid follicles, with very large and active germ centres. The Malpighian bodies of the spleen were large and prominent—an enlargement mainly due to the size of the germ centres, which were active, cellular and hyperplastic, as in the case of the lymph-glands. None of the other animals, nor a control *rhesus* which had been examined a short time before, showed any such change.

Experiment 2.—Male *rhesus*. This experiment was valueless except as a control, in that the monkey had to be killed on the tenth day, as it was suffering from *Staphylococcus aureus* suppuration of the abdominal wound. Splenic implantation only had been carried out, and sections of this organ showed a small area of necrosis and hæmorrhage immediately under the capsule. Otherwise the spleen, as also the lymph-glands, appeared normal microscopically, and were in striking contrast to those of the first monkey.

Experiment 3.—Male *rhesus*. In this experiment a small quantity of thick emulsion of Hodgkin gland-tissue in saline was injected with a syringe into the spleen, and into the substance of two large lymph-glands in the ileocolic angle. The animal made a good recovery and remained well. One year later the abdomen was reopened. There were extensive adhesions around the spleen, and a large round nodule ¼ in. in diameter was present at the site of lymph-gland inoculation. This was excised, and found to be an abscess containing a small nematode. This monkey was killed after two years, and showed no pathological changes at autopsy.

Experiment 4.—Adult male bonnet monkey. Here the spleen and a lymph-gland were injected with a coarse emulsion as in Experiment 3, and in addition a few small chips of Hodgkin tissue were implanted into a second lymph-gland. The animal made a good recovery and remained well. Four and a half months later the abdomen was opened and the spleen excised. No enlarged glands were seen. A large amount of material from the same case of Hodgkin's disease was again implanted and injected into lymph-glands, liver, intestinal wall and rectus abdominis muscle, while pieces of tissue were left free in the peritoneum. The animal appeared to be none the worse for this operation, and remained in good health until it was killed 2 years and 8 months later. At autopsy there was no evidence of lymph-gland enlargement. A small necrotic focus was found in the liver. Microscopic examination of both spleen and lymph-glands revealed no pathological changes.

SUMMARY AND CONCLUSIONS.

Inoculation and implantation experiments with lymph-glands from two active cases of Hodgkin's disease were carried out on three *Macacus rhesus* monkeys and one bonnet monkey. One *rhesus*, after 4½ months, showed an unusual degree of simple hyperplasia of the lymphoid follicles and of the

Malpighian bodies of the spleen, especially of the germ-centres in each instance, together with a peculiar giant-cell reaction, probably of foreign-body type, around the implanted material. The remaining experiments yielded completely negative results, the bonnet monkey being under observation for three years and one *Macacus rhesus* for two years before they were slain for examination.

These experiments afford further proof that whether or not Hodgkin's disease is an infective granuloma, it is at least not due to a virus capable of reproducing the disease in monkeys.

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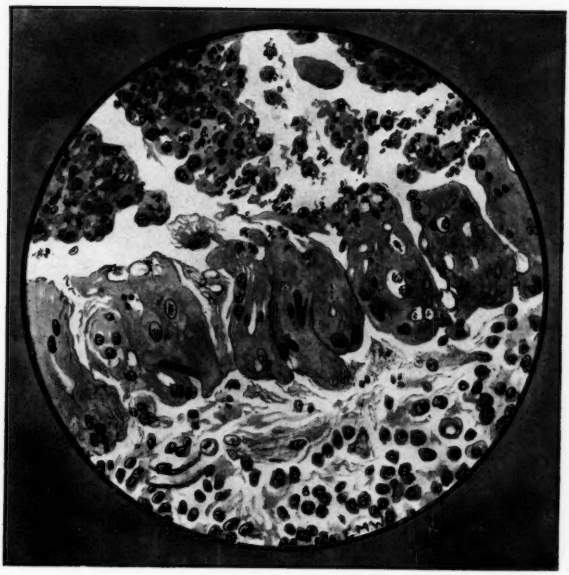
DESCRIPTION OF PLATE.

Portion of wall of "abscess"-cavity in a lymph-gland into which Hodgkin gland-tissue had been implanted four and a half months before. The contents of the abscess, seen in the upper part of the field, consist of cells and cellular *débris* including many neutrophil polymorphonuclear leucocytes. The wall of the abscess-cavity is lined by a palisade-like formation of multinucleated giant-cells, presumably of the nature of a foreign-body reaction. × 350.



Stewart and Dobson.

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THE GROWTH REQUIREMENTS OF HÆMOLYTIC INFLUENZA BACILLI, AND THE BEARING OF THESE UPON THE CLASSIFICATION OF RELATED ORGANISMS.*

PAUL FILDES.

From the Bacteriological Department, London Hospital.

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In a previous paper (Fildes, 1923), it was shown that of the organisms which are commonly grouped together as "hæmoglobinophilic," only two are known which have the characteristic of being dependent for growth upon blood-pigment or upon a substance acting in a similar way. These two organisms are *B. influenzæ* itself and *B. hæmoglobinophilus canis*.

In a classification, therefore, which is based upon the blood-pigment effect, loosely described as "hæmoglobinophilia," these two organisms only may be included.

In the same paper it was shown that the reactions of Koch-Weeks' bacillus do not differ from those of *B. influenzæ*, and thus these organisms may be looked upon as being identical.

The two members of the hæmoglobinophilic group differ in their reaction towards the second, so-called "vitamin" growth factor of the influenza bacillus. Whereas *B. influenzæ* will not grow except in the presence of this factor, *B. hæmoglobinophilus canis* will grow without it. From consideration of the nature of the "symbiosis phenomenon," it was deduced that *B. hæmoglobinophilus canis* could synthesize the V factor and so did not require it to be added, while *B. influenzæ* was unable to do this. Thus the fundamental difference between these two organisms lay in the capacity of one to synthesize the V factor and in the incapacity of the other.

I have recently had the opportunity through the kindness of Dr. Martin Kristensen of Copenhagen to test the behaviour of 14 strains of "hæmolytic influenza bacilli" towards the two growth factors of the influenza bacillus. The strains were numbered 1–16, Nos. 13 and 15 being omitted. They are preserved in the National Collection of Type Cultures, London.

These bacilli were first described by Pritchett and Stillman (1919), and are capable of being isolated from a considerable proportion of normal throats.

GENERAL CHARACTERS OF THE HÆMOLYTIC INFLUENZA BACILLI.

Judging from the 14 strains under examination these bacilli appear to constitute a well-defined group. The chief data of interest are as follows:

* A report to the Medical Research Council.

Morphologically there are few points of absolute distinction from influenza bacilli, but on the whole they tend to be stouter, and, especially in liquid media, are found in definite chains.

Culturally they differ from influenza bacilli chiefly in their more profuse growth on blood-agar and in their behaviour in liquid media. While *B. influenzæ* nearly always grows diffusely, these bacilli develop stringy, flocculent masses in a clear medium. In liquid media they grow with great rapidity, and in all media die out much sooner than do influenza bacilli.

The *fermentation reactions* are distinct. Tested in bactopeptone water containing yeast extract, they readily ferment glucose, saccharose and maltose, but not lactose, dulcite or mannite, whereas *B. hæmoglobinophilus canis* ferments glucose, saccharose and mannite, but not lactose, dulcite or maltose. One strain (No. 11) of the hæmolytic bacilli failed in repeated tests to ferment maltose. *B. influenzæ* does not ferment any of these sugars. As pointed out by Kristensen (1922), reports of sugar fermentation by *B. influenzæ* on agar media are unsatisfactory.

THE GROWTH REQUIREMENTS OF HÆMOLYTIC INFLUENZA BACILLI.

In Kristensen's (1922) monograph it will be seen that these bacilli are "decidedly less hæmoglobinophilic than Pfeiffer's bacillus." Some are capable of growth on ordinary agar. But they resemble *B. influenzæ* in being to a certain degree stimulated by other organisms. Some strains are stimulated strongly, others not at all.

In the present investigation the growth requirements of these bacilli were tested by the technique already described (Fildes, 1922, 1923). The basis of the medium was bactopeptone water. Filtered yeast extract was added as a test for the V requirement and a pure hæmatin solution as a test for X requirement. The inoculations were made in such a manner that transference of blood-pigment was reduced to a minimum. In order to obtain constant results, *B. influenzæ* must be inoculated extremely sparsely, otherwise sufficient blood-pigment will be carried over to give a slight growth in yeast alone. On the other hand, *B. hæmoglobinophilus canis* and the hæmolytic bacilli must be inoculated rather more heavily from actively growing cultures, since the growth energy of some strains is small under the conditions of the experiment.

Three strains of *B. influenzæ* and one of *B. hæmoglobinophilus canis* were always included as controls.

The growth requirements of all strains of hæmolytic bacilli except those of No. 11 were the same, as follows:

	Peptone water plus—			
	Nil.	Hæmatin.	Yeast.	Hæmatin + yeast.
B. influenzæ	0	0	0	+
B. hæmoglobinophilus canis	0	+	0	+
Hæmolytic influenza bacilli	0	0	+	+

+ indicates that the bacilli grew copiously when subcultured after 2 days' growth from the tube in question to peptic blood-agar (Fildes, 1920). Estimates of growth depending upon opacity are unsatisfactory owing to a frequent development of deposits in the yeast tubes. 0 = no growth upon subculture of one loop (0·003 c.c.).

In connection with these results, it is, of course, possible that growth takes place with one factor by reason of a transference of a minute quantity of the other factor with the inoculum—yeast in the case of *B. hæmoglobinophilus canis* and hæmatin in the case of the hæmolytic bacilli. This point was investigated by serial transfers through yeast or hæmatin as the case might be. The transfers were made with a small loop having a capacity of 0·003 c.c. It was found that all strains of the hæmolytic bacilli could be carried through yeast for at least 7 subcultures with undiminished vigour. Similarly *B. hæmoglobinophilus canis* could be carried through hæmatin without yeast for seven generations.

With regard to *B. influenzæ* the matter is more complicated. It is not at all uncommon to find, particularly with No. 293 of the National Collection of Type Cultures, originally isolated in 1919, that 50 or more colonies develop from the loop of material taken from the apparently ungrown yeast tube. This number indicates a definite growth, and, furthermore, if the loop is transferred to another yeast tube, a slight multiplication may even be found in this also. It is possible, then, that a multiplication of *B. influenzæ* may take place in the absence of hæmatin, but, on the other hand, it is more probable that these growths are due to hæmatin actually adsorbed to or taken up into the bodies of the bacilli and transferred with them.

Thus the growth requirements of the hæmolytic bacilli (except No. 11) are quite different from those of the other bacilli tested. While *B. hæmoglobinophilus canis* requires hæmatin only, the hæmolytic bacilli require yeast only, but *B. influenzæ* demands both of these factors.

THE SYMBIOSIS PHENOMENON WITH HÆMOLYTIC INFLUENZA BACILLI.

The dependence of the hæmolytic bacilli and of *B. influenzæ* upon yeast suggests the possibility that in each case the reason is the same, namely that the hæmolytic bacilli are incapable of synthesizing the V factor themselves. If this is the case, they should be stimulated in the symbiosis phenomenon in the same way as is *B. influenzæ*. As mentioned, Kristensen (1922) found that this was the case with some but not with all strains.

Stimulation of Hæmolytic Bacilli by Staphylococcus aureus.

The symbiosis test was carried out as previously. The basal medium contained agar, salt, bactopeptone and hæmatin. This was set in slopes, diffusely inoculated with the organism to be tested, and finally inoculated along one extreme edge with *Staphylococcus aureus*. Under such circumstances *B. influenzæ* grows with its largest colonies in the vicinity of the staphylococcus streak, while the growth fades away to nothing towards the other side of the slope.

When the fourteen hæmolytic strains were tested, two (Nos. 7 and 10) showed a well-marked positive symbiosis effect, but the other strains did not grow at all. It was clear that most of these strains must have a sufficient quantity of V factor added to the medium, to allow them to grow slightly, before the stimulating effect of the staphylococcus could be judged. The organisms died out before the V factor was elaborated by the staphylococcus.

On repeating the tests with the addition of peptic blood (Fildes, 1920), it was found very difficult to arrange satisfactorily the amount to be added. If too much V factor is added the growth becomes so profuse that no stimulation can be detected. Each strain appeared to require different amounts at different times, and it was only by continually repeating the tests with quantities varying from 0·05 to 0·5 per cent. that positive results were obtained. Nos. 7 and 10 were most consistently positive, but marked reactions were also obtained with Nos. 1, 2, 3, 4, 5, 8 and 11. With the other strains the results were very variable, but all showed slight stimulation on some occasion.

The stimulating action of staphylococcus was also tested by using filtered liquid cultures of the coccus. In this way the absence of V factor during the lag period could be avoided.

A flask of bactopeptone water was inoculated with staphylococcus, grown for two days, filtered through a Berkefeld N candle and tubed. These tubes and peptone water controls were then inoculated with the 14 strains of hæmolytic bacilli and growth took place in the filtrate in every case, feebly in some, but strongly in others.

It may therefore be concluded that the V factor elaborated by staphylococci is capable of supplying the missing growth factor to hæmolytic influenza bacilli in the same way as to *B. influenzæ*, and that, so far as the test is technically possible, the symbiosis phenomenon is positive with these organisms.

Stimulation of Hæmolytic Bacilli by B. hæmoglobinophilus canis.

The same result may be observed by using the V factor synthesized by *B. hæmoglobinophilus canis.*

For the symbiosis test the same technique was used, namely double inoculations on agar containing small quantities of peptic blood, but hæmatin was also added to allow a good growth of *B. hæmoglobinophilus canis.*

Again, Nos. 7 and 10 gave the most pronounced positive results, but all others reacted to a greater or less extent, on the whole less obviously than with staphylococcus.

When using a filtrate of a 48-hour culture of *B. hæmoglobinophilus canis* in peptone water *plus* hæmatin, growth was only observed with Nos. 1, 4, 6, 7, 8, 10 and 16.

Thus *B. hæmoglobinophilus canis* is capable of stimulating the growth of the hæmolytic bacilli in the same manner as staphylococcus but possibly to a less extent.

Lack of Stimulation of B. influenzæ *by the Hæmolytic Bacilli.*

All strains of the hæmolytic bacilli were tested by the same technique upon several strains of influenza bacilli, and in no instance did any stimulation of the influenza bacilli take place.

It thus follows that the hæmolytic bacilli do not synthesize the V factor required by the influenza bacillus. This would be surmised from the fact that they require the addition of V factor for growth themselves.

It will be remembered that Davis (1921[1]) has suggested that failure to stimulate *B. influenzæ*, combined with a positive reaction in the symbiosis test, is an indication that the bacillus in question is an influenza bacillus, but it it clear that this rule is not of general application.

From these experiments the conclusion may be drawn that the hæmolytic bacilli resemble *B. influenzæ* in being unable to synthesize the V factor, although they differ in that they do not require the X factor (blood-pigment).

DISCUSSION.

Rivers (1922[2]) and Rivers and Bayne-Jones (1923) have described a bacillus (*B. parainfluenzæ*) in two human cases and in cats which appears to have the same growth requirements as the hæmolytic influenza bacilli but is not hæmolytic. Rivers (1922[1]) also found that *B. hæmoglobinophilus canis* depended upon hæmatin only. Rivers and Bayne-Jones classified these two organisms with *B. influenzæ* as hemophilic bacilli differing in their growth requirements.

The question arises whether the dependence upon blood-pigment or the possibly unrelated inability to synthesize V factor is the more important from the point of view of the classification of the organisms which are typified by *B. influenzæ*, or whether it may be supposed that both characters acting together are fundamental as a criterion of the typical member of the group.

If the two factors, although perfectly distinct, have no connection with one another—and the possibility had been rather favoured by Rivers (1922[1])—it is difficult to agree to a classification which embraces the three organisms. If the classification depends upon "hæmoglobinophilia," then the hæmolytic bacilli and Rivers' *B. parainfluenzæ* are excluded.

On the other hand, if it is allowed that the two factors are in some way related and interdependent—a view favoured by Davis (1921[2]) and Fildes (1921)—it is possible to look upon *B. hæmoglobinophilus canis* as aberrant in that it has developed the power itself to synthesize one of the essential factors —the V factor—while, on the other hand, the hæmolytic bacilli may have developed the power themselves to supply whatever the X factor may represent.

If this argument is correct, it would be justifiable to class together these three bacilli, not in a hæmoglobinophilic group, but in a group a designation for which must await further investigation.

The possibility that in the course of their evolution the hæmolytic bacilli have developed an independence of hæmatin is to some extent supported by the fact that their independence does not seem to be absolute in all cases. Strain No. 9, for instance, is carried through yeast with much greater difficulty than are strains Nos. 7, 10, 16 and others. The growth is usually greater when hæmatin also is added, and may take place when the yeast-tube will not grow with the same inoculum. Again, as has been noted, strain No. 11 seems to represent a "cross" between *B. influenzæ* and the hæmolytic bacilli. It resembles *B. influenzæ* in having the same growth requirements and in growing diffusely, but differs in being hæmolytic and in fermenting glucose and saccharose. On the other hand, it resembles the hæmolytic bacilli in

being hæmolytic and in fermenting sugars, although not maltose. In the case of *B. influenzæ* itself, there appears to be little doubt that some strains can grow with less hæmatin than others, and it is even possible, but not probable, that some multiplication may take place in the absence of hæmatin.

It is therefore suggested that the three organisms under discussion have a certain relationship depending upon the extent to which they utilize the X factor or synthesize the V factor: that typical strains of these three organisms illustrate the three main divisions to the most pronounced degree, though other strains occur which occupy intermediate positions in the group. Among these may be reckoned strain No. 11 and Rivers' *B. parainfluenzæ*. It would appear adequate to refer to an "influenza group" all such bacilli with growth requirements wholly or partly similar to those of *B. influenzæ*.

CONCLUSIONS.

1. The growth requirements of Danish hæmolytic influenza bacilli differ from those of *B. influenzæ* or *B. hæmoglobinophilus canis*. They are indifferent to the blood-pigment factor but demand the V factor.

2. The behaviour of these bacilli in the symbiosis test indicates that they are incapable of synthesizing the V factor.

3. Thus a group of bacilli is shown to exist which has the following characters:

	Blood-pigment factor required.	Inability to synthesize V factor.
B. influenzæ	+	+
B. hæmoglobinophilus canis	+	−
Hæmolytic influenza bacilli	−	+

4. It is suggested that this grouping may form the basis for a classification, *B. influenzæ* being the type, and the others related organisms which have the power to supply one of the factors themselves. Between these extremes intermediate varieties exist. All such bacilli may be referred to as "influenza group bacilli" rather than as "hæmoglobinophilic."

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BEHAVIOUR OF DEPANCREATIZED DOGS KEPT ALIVE WITH INSULIN.

F. N. ALLAN, D. J. BOWIE, J. J. R. MACLEOD, AND W. L. ROBINSON.

From the Departments of Physiology and Pathology, University of Toronto.

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IN depancreatized dogs kept alive by insulin and used by one of us (Allan, 1924) for the purpose of measuring the glucose equivalent of this hormone, it was observed that although the animals remained in excellent nutritive condition for a long time, four of them ultimately developed symptoms which ended fatally after periods varying between one and seven months. On post-mortem examination the liver was found to be the seat of very extensive fatty changes in all animals, but only in one of them was there evidence of toxic degenerative changes in the kidneys. Although it will take some time before it will be possible to complete this investigation, particularly with regard to its chemical aspects, the results so far obtained are of such a definite nature that we have thought it advisable to communicate them briefly and to suggest a possible explanation for them.

DESCRIPTION OF ANIMALS.

Dog H. 2 mo

Female, weighing to start 9·2 kg., was depancreatized on February 13th, 1923, after which it was given insulin twice daily, and fed at first with various types of diet and later with lean meat and cane-sugar. The amount of meat at first was 500 gm. daily along with 100 to 150 gm. cane-sugar. Towards the end of the observation the meat was increased to 900 gm. Glycosuria was present throughout the observation, the degree of which varied according to the dose of insulin. The animal remained in good condition until April 12th (two months after the operation), when, in contrast to its previous behaviour, it appeared depressed and apathetic, and took little food. The rectal temperature was 40° C. Next day the dog refused food, and jaundice was noticed in the sclera and the skin inside the ears. Bile-pigment was found to be present in the urine, the volume of the latter becoming less and less, so that the concentration of bile rose very greatly. Unfortunately the urine was not tested for protein. On April 14th the dog was very weak and apathetic. During the day it became extremely feeble and the respirations rapid and somewhat distressed; it died during the following night. In the cage were found dark semi-fluid fæces containing a small amount of red blood.

Post mortem.—The peritoneal cavity was found to contain a small amount of yellowish fluid. The liver was large and very friable and was of a pale yellowish nutmeg appearance. No obstruction could be detected in the biliary tract. The gall-bladder contained about 5 to 10 c.c of bile. The kidneys and spleen appeared to be normal.

Microscopic report.—Sections of the liver showed marked degenerative changes in the cells about the central vein (see Fig. 1). The cells were isolated in small groups, swollen, and had an acidophilic granular cytoplasm, with numerous vacuoles which stained for fat. The nuclei of these cells for the most part did not stain; in other cases they were pycnotic. There were numerous red blood-cells in the sinusoids of this area. Endothelial leucocytes filled with a brownish pigment (bile-pigment) could be seen with the high power. The degenerated areas represented, usually, about half of the lobule. The liver-cells in the outer portion of the lobules were swollen, and contained either a single moderate-sized vacuole or a number of small vacuoles which stained deeply for fat. The nuclei of these cells stained well. The bile-ducts, bile-capillaries and blood-vessels showed nothing abnormal. Glisson's capsule was infiltrated with a few plasma-cells. It was certain that the liver contained a great excess of fat, but no chemical analysis was made to determine the amount.

In sections of the kidney the changes in the glomerular tufts were not significant. Some of the tufts completely filled Bowman's capsule. In other cases they were retracted and their capsule filled with a very finely granular *débris*, or with small masses of what appeared to be swollen and degenerated epithelial cells, which had desquamated from the lining of Bowman's capsule. No inflammatory cells could be seen. The cells lining the proximal convoluted tubules and the distal tubules showed very marked degenerative changes. This was more marked in the proximal convoluted tubules. Many of these degenerated cells had desquamated into the lumen and were forming granular casts. The fat stains revealed many fine droplets of fat in the degenerating cells. The cells lining the loop of Henle and the collecting tubules showed some slight degenerative changes, with fair-sized fat droplets in the cytoplasm of most of the cells. In the stroma there was a fair amount of œdema, (evidenced by the wide separation of the stroma-cells), and hæmorrhages of moderate size occurred here and there. There was no evidence of bile-pigment.

Dog I. 4710

Female, weighing to start 6·25 kg., was depancreatized on April 5th and made excellent recovery from the operation under the influence of insulin. It was then fed with a daily ration of 600 gm. lean meat (corresponding to about 9 gm. nitrogen) and 100 gm. cane-sugar. The urinary nitrogen ranged from 6·6 gm. to 9·5 gm., so that less than 50 per cent. of the ingested protein was being absorbed. Some sugar was constantly present in the urine, but the amount varied from day to day according to the dose of insulin. Excellent nutrition was maintained and the body-weight for some time increased slightly. The animal was in good spirits and apparently normal in every way, taking its food with relish. The fæces were normal in appearance. During July it was observed that the hair was falling out very rapidly, the skin being dry and scaly with gradually increasing patches of eczema, and catarrhal conjunctivitis developed. On July 28th the appetite began to fail and on August 1st severe diarrhœa set in, followed in a few days by vomiting and rapid loss in body-weight. These symptoms persisted until

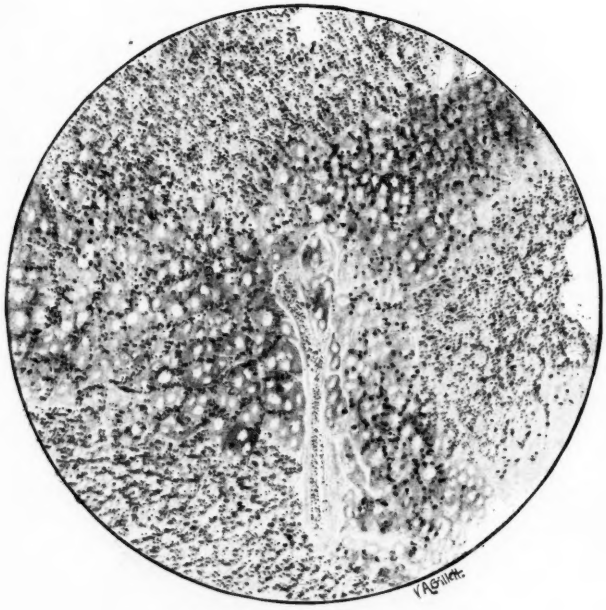

FIG. 1.

Section of liver of Dog H, which died two months after pancreatectomy although treated with insulin. Stained with hematoxylin and eosin. × 100.

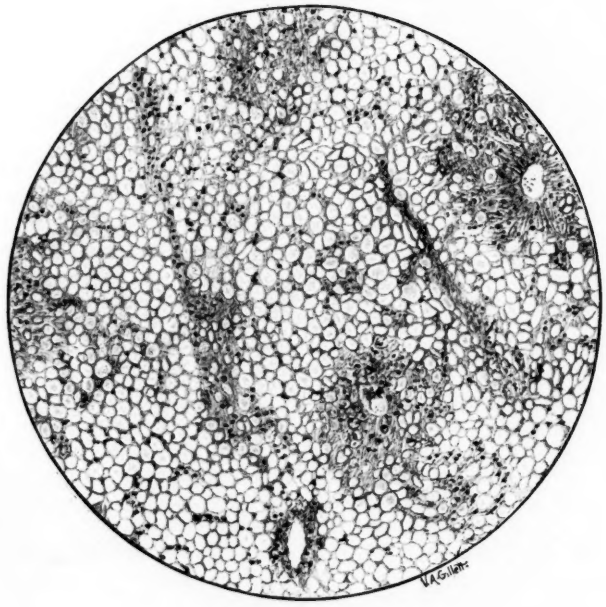

FIG. 2.

Section of liver of Dog J, which died seven months after pancreatectomy although treated with insulin. Stained with hematoxylin and eosin. × 100.

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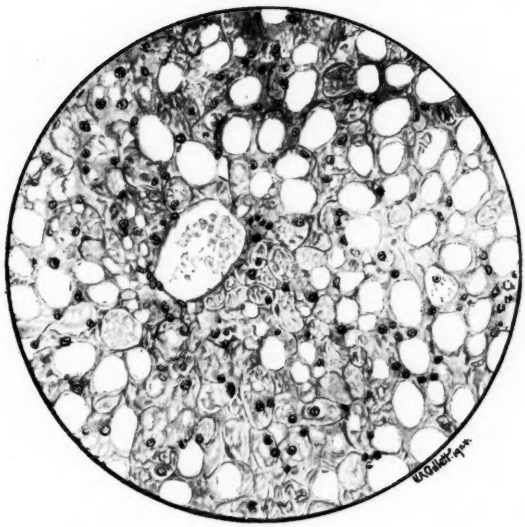

FIG. 3.

Part of the same liver as that shown in Fig. 2, but more highly magnified. Stained with hematoxylin and eosin. × 400.

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the death of the animal, which occurred on August 7th, four months after pancreatectomy. The fæces passed just before death were very dark in colour and semifluid in consistence like molasses. The weight of the animal at death was 5·33 kg.

Post mortem.—The liver was very friable, weighed 616 gm., and was of a mottled orange-yellow colour. The gall-bladder was half filled with a thick, very dark fluid. On section the organ was seen to be loaded with fat, especially towards the periphery. The kidneys appeared somewhat enlarged, and on section whitish areas (about the size of a pea) were seen in the cortex. There were very few adhesions in the region of the pancreas and no trace of this gland could be made out.

Microscopic report.—The liver-cells about the periphery of the liver-lobules, with appropriate stains, were seen to be filled with fat. For the most part it appeared as a single globule pushing the nucleus to one side. The liver-cells about the central vein contained smaller globules of fat. Many of these cells were undergoing degeneration, as evidenced by the pycnotic changes in the nuclei and the granular appearance of their cytoplasm. In a few areas deposits of bile-pigment were found in small brown masses, or occurring as wormy deposits either within cells or within short segments of bile canaliculi. These deposits were quite infrequent, and, taken as a whole, the section did not suggest there had been much obstruction to the canaliculi in the liver.

The sinusoids were more or less completely collapsed, due to the pressure of the swollen liver-cells.

The kidney exhibited a fairly marked acute pyelonephritis, as evidenced by the large collections of polymorphonuclear leucocytes in the stroma with abscess formation.

Serial sections of the duodenum revealed a piece of what appeared to be normal pancreatic tissue situated in the serous coat and measuring about 1 mm. by 3 mm. One could not make out any islet tissue as being present.

Sections of the heart muscle, thyroid and suprarenal glands were apparently normal.

Dog J. 6210

Female, weighing to start 7·5 kg., was depancreatized on April 16th, from which it recovered rapidly under insulin, which was given twice daily. It was fed on 500 gm. lean meat and 100 gm. cane-sugar. On April 29th the animal lost its appetite and vomited when it was persuaded to take food. Bile and a small amount of protein appeared in the urine. A blood culture was negative. These symptoms gradually disappeared, the animal regained its appetite and remained in excellent condition on the above diet with insulin, some degree of glycosuria being constantly present. Early in November the cane-sugar was removed from the diet, with the result that the body-weight rapidly declined and the animal became very weak and refused its food. On November 17th bile appeared in the urine and some fresh blood in the fæces. No jaundice could be detected. Attempts at forced feeding with meat and cane-sugar were unsuccessful because of vomiting; the animal became rapidly weaker and died on November 21st. The average daily excretion of nitrogen in the urine of this animal varied between 7·2 and 11·0 gm.; since about 16 gm. was

ingested daily, the balance indicates a better absorption of protein than in dog I.

Post mortem—The body was still warm when the post-mortem examination was made and there was no *rigor mortis*. The weight was 5·11 kg. No fat could be detected in the subcutaneous tissues and the muscles were very greatly wasted. The peritoneum was normal except for a few adhesions at the site of operation and between the liver and duodenum. No trace of pancreatic tissue could be detected and the duodenum was perfectly healthy. The liver weighed 650 gm. (12·65 per cent. of the body-weight), and was of a pale yellow colour with a nutmeg appearance. It was very friable and left a grease stain on the knife. The gall-bladder contained about 20 c.c. of bile, which could be squeezed into the intestine. The kidneys were small and of a dark red colour, and on the surface were numerous paler depressions. The capsule stripped with difficulty. On section the dark red colour of the cortex was in sharp contrast with the pale medulla. Fine granular calculi were present in the pelvis. The urinary bladder was very much thickened, and there was marked evidence of chronic cystitis. Calculi were also present. The heart and the lungs were normal, the former, however, being very flabby.

Microscopic report—In sections of the liver (Fig. 2) the cells about the central vein were slightly swollen, a little more granular than normal, and contained an occasional small globule of fat. In many cases the nuclei showed pycnotic changes, while others showed fragmentation. About these areas there was a small infiltration of polymorphonuclear leucocytes. Throughout the greater part of the lobule the liver-cells were large, and occupied by a single large globule of fat which pushed the nucleus to one side.

Scattered irregularly through the section one found liver columns in which bile canaliculi were demonstrated by the presence of short wormy masses of bile-pigment. These pigment deposits were not continuous along the liver columns, but occupied only short segments of it. It would appear that the distal portion of the liver columns had suffered swelling of the liver-cells to a degree causing stasis of the bile secreted in the more proximal portions of the bile canaliculi.

In the kidneys the glomeruli appeared normal, and the cells of the proximal convoluted tubules, while swollen, showed no evidence of degeneration. There were one or two small areas of polymorphonuclear infiltration of the medulla.

The spleen and heart muscle appeared normal. No evidence of pancreatic tissue could be found in serial sections of the duodenum.

Chemical report.—The moist liver contained 35·9 per cent. of fat with an iodine value of 62·2 to 65 and 1·8 per cent. of glycogen. The muscles contained 0·81 per cent. of glycogen and the heart 0·375 per cent.

Dog D.

Besides the three animals, H, I and J, described above, there was a fourth one, D, in which the symptoms of hepatic involvement developed at a much earlier date following the pancreatectomy. Indeed, this was the first animal in which these appeared, and, their possible significance not being recognized,

the pathological condition responsible for them was not so carefully studied. This animal, weighing 12 kg., was depancreatized on November 23rd, 1922. Small amounts of insulin were given during two days following the operation and then discontinued until the 28th. During this time the diabetic symptoms became very marked, but they disappeared after the insulin was again started, and the animal took food greedily. On December 10th the animal began to refuse food and it lost in weight. On December 13th bile was detected in the urine and jaundice became marked, particularly in the ears and sclera. The animal was moribund on December 20th and was chloroformed.

To recapitulate, the deposition of fat in the liver in the three insulin-treated animals is most marked in the outer portion of the lobules, although much is also present in the cells near the centre. In the cells of this region a certain amount of degeneration is evident, and in those nearer the periphery of the lobules many of the nuclei do not stain, and the protoplasm that remains in the cells is highly granular in appearance. Conspicuously more fat is visible in these than in the livers of two dogs that died in four and fourteen days respectively after pancreatectomy without being treated with insulin. A distinct difference also exists in the distribution and appearance of the fat deposits in the liver in the two types. In the non-insulin livers the fat deposits are decidedly richer in the cells toward the centre of the lobules—where also the globules are larger—than in the cells toward the periphery, and the central cells are swollen and compress the sinusoids. In the insulin-treated livers the fat is so abundant (*e.g.* 35·9 per cent. in dog J) and it is deposited in such large globules that the sections appear like those of adipose tissue, except for a few swollen liver-cells that remain near the sub-lobular veins. In the absence of further chemical evidence we cannot be certain, however, that there is always more fat in the livers of the animals that have died after prolonged treatment with insulin than in those not treated. We hope to report on this part of the work at a later date. Whether or not there is a marked difference in the amount and distribution of fat in the liver under the two conditions, the significant fact revealed by these investigations is that fatal symptoms, indicating a breakdown of the hepatic function associated with severe fatty infiltration of the liver, occurs in depancreatized dogs that have been treated daily with large amounts of insulin for from two to seven months.

DISCUSSION.

Since the administration of insulin along with carbohydrate promptly removes the excess of fat which otherwise appears in the liver in a few days following pancreatectomy, the reappearance of the fat observed in these investigations is difficult to understand. Several depancreatized animals have also been kept, with or without insulin, during shorter periods of time than was the case with those reported above. Not being useful for the purpose of determining the glucose equivalents of insulin, they were used in the investigation of various other problems, such as the behaviour of the respiratory exchange, and on their deaths portions of the liver, muscles, etc., were removed to determine the percentage of fat and glycogen. The following table gives a summary of the results:

TABLE I.

Dog.	Date.	Organ.	Fatty acid, per cent.	I-value.	Remarks.
25	Dec. 3rd	Liver	11·21	81·2	Depancreatized 4 days. No insulin.
		Blood	(5·68)	78·65	
		Kidney	4·55	81·8	
		Muscle	4·91	71·9	
27	Dec. 4th	Liver	7·69	81·63	Depancreatized 3 days. No insulin. D:N ratio (without food) of bladder urine, 2·86. Glycogen in liver, 0·45 per cent.
		Blood	1·75	72·6	
		Kidney	3·68	100·0	
		Muscle	5·2	72·01	
26	Dec. 20th.	Liver	3·85	89·16	Depancreatized 17 days. No insulin until 3 days before death, when much sugar was also given. Glycogen in liver, 1·79 per cent.
		Blood	0·57	104·9	
		Kidney	4·45	104·4	
		Muscle	1·17	116·5	
		Omentum	—	79·6	
24	Dec. 20th.	Liver	19·18	70·2	Depancreatized Nov. 22nd and kept on insulin and pancreas till Dec. 18th. Insulin then discontinued and animal rapidly went downhill, dying on Dec. 20th following a respiratory experiment. Insulin given a few hours before death.
		Kidney	3·63	88·03	
		Muscle	6·11	76·17	

Dogs 25 and 27 serve as useful controls to show the percentages of fat and glycogen which occur in the various organs in 4 and 3 days respectively, following pancreatectomy when no insulin is given. Dog 26, weighing 7·2 kg. was depancreatized on December 4th. After recovering from the operation it was fed on lean meat, and on December 13th and 14th the D:N ratios were 2·87 and 3·16 respectively. On December 18th (14 days after the operation) the animal was greatly emaciated but in fairly good general condition. It was now fed with sugar, and large doses of insulin were administered until December 20th, when it was killed. It will be observed that the fat in the liver was only 3·85 per cent. and in the blood 0·57 per cent., which are both somewhat below the usual values for normal dogs. The iodine value of the fat of the liver was somewhat above that of the omentum, but below that of the blood, kidney and muscles.

Dog 24 is of particular interest. The animal after pancreatectomy was kept for nearly four weeks on a diet of meat, to which fresh pancreas was sometimes added and cane-sugar, and was injected twice daily with insulin. It remained in excellent condition, the only noteworthy fact being that the fæces were sometimes tinged with fresh blood. After this time, December 18th, the insulin was discontinued for the purpose of determining the behaviour of the respiratory metabolism, but on the day following the discontinuance it was found that the animal had become weak and that it refused food. On December 20th the animal was very weak and became rapidly more so during the day, becoming semi-comatose during the afternoon with rapid shallow respiration. A marked reaction for ketone bodies was obtained in the urine. Insulin was administered at 5.30 p.m., but the animal died during the night. The very rapid development of symptoms of great bodily weakness, of inability to take food, and, later, of coma and polypnœa, were apparently dependent upon the withdrawal of insulin, and we have observed the same

results to follow in two other animals similarly treated. There is no doubt that discontinuance of insulin treatment in depancreatized animals that have kept in excellent condition while it was being given, along with meat and sugar, results in an immediate breakdown in metabolism causing symptoms extremely like those of coma, as observed in human diabetes. This important and unexpected observation demands further investigation, and with this we are engaged at present. From the analyses of the liver it will be seen that a very large amount of fat with an iodine value close to that of the omentum had accumulated in the liver. Since the animal was not kept in its cage during the day preceding its death no urine was collected. Attempts to measure the respiratory exchange on the second day after discontinuing the injections of insulin were frustrated because of the polypnœa and the extremely weak condition of the animal.

Similar fatty infiltration of the liver occurs whenever the function of the cells is seriously interfered with, as, for example, by toxic agencies, phosphorus, arsenic, etc. The question therefore arises whether some toxic substance may not be responsible for the fatty changes observed in insulin-treated dogs. If such be the case the toxic substance must either be introduced from without with the injections of insulin, or it must accumulate in the body as a result of some fault in metabolism. With regard to the former possibility, it must be remembered that insulin solutions contain 0·3 per cent. trikresol as a preservative, and since two injections of 1 to 2 c.c. each were given daily, as much as 1800 mg. were often administered during one month. This possibility is difficult to control. Injections of trikresol in normal animals has not been observed by us to cause any symptoms like those described above; but this is not a perfect control, because such substances might have a greater toxicity in the presence of the slight degree of hyperglycæmia and glycosuria, which, as already has been explained, were allowed to be present in these animals. If we can rule out the possible toxic influence of the preservative, it is difficult to see how any other directly toxic substance should be present in the insulin preparations. In their present state of purity there is only the faintest trace of a biuret test, Millon's and the xanthoproteic tests being negative, and the nitrogen content is only about 0·03 mg. N per (clinical) unit. The improbability that the injections of insulin are directly responsible for the toxic effects is further sustained by the fact that none such have been observed in patients who have been under insulin, and there are now several cases in which this has been continued for nearly two years. We are inclined to conclude, tentatively at least, that the condition, whether toxic or not, which is responsible for the infiltration arises within the animal. This condition probably develops gradually, and is not revealed by any symptoms until so much damage is done to the liver that acute symptoms of a breakdown of the hepatic function set in. We are attempting to detect this onset in animals at present under observation partly by observing the blood at regular frequent intervals for the presence of bilirubin and for the amount of fat, and partly by measuring the rate of excretion of phenoltetrachlorphthalein as recommended as a test of hepatic function by Rosenthal. Until this work is completed we cannot say whether the upset of hepatic function, which is apparently the cause of death, is one of gradual or of sudden development.

If the condition in the liver is the result of a general intoxication it would be expected that the kidneys also would be the seat of serious damage. In one of the animals (H) this was indeed the case, marked nephrosis being evident. In the other two, however, the kidneys, although pathological, did not exhibit evidence of a change due to toxic substances. In one of these cases (J) collections of lymphocytes were present in the papillary zone and in the other (I) acute pyelonephritis was evident. It is interesting to note also that it was in the animal in which the toxic symptoms supervened after two months, and not in those of four and seven months, that the nephrosis was present. Examination of other organs, such as the spleen, thyroid and suprarenals, and of muscle-fibre (skeletal and cardiac), did not reveal evidence of a toxic condition in any of the animals.

Although in the light of these observations it is impossible to be certain that the fatty changes in the liver in all three cases may not have been the result of a general intoxication, there is evidence that the primary fault was one affecting the liver, the nephrosis being then secondary to the toxic state which this would induce.

In some way or another the continued absence of the pancreas leads to a pathological condition in the liver-cells, and as a result fat accumulates in them. Since there is no evidence that a similar condition becomes developed in diabetic patients treated with insulin, it is possible that the pancreas, quite apart from its function in producing this hormone, also produces some other internal secretion necessary for the physiological integrity of the liver. Since so much fat accumulates in the cells of this organ, it is natural to consider the possibility that this may have to do with the chemical changes which fatty acid undergoes in the liver-cells, and which, as Leathes and others have shown, are an essential step in their preparation for oxidation (desaturation). In this connection one has to bear in mind that between the most severe case of diabetes in man and a depancreatized dog treated with insulin there exists a great difference, in that there still remains in the former some functioning pancreatic tissue which can produce, among other things, lipase. Most of this is no doubt secreted with the pancreatic juice, but some may be absorbed into the blood, and therefore furnish the liver and other organs with the supply of this enzyme which they require in the mobilization of fat.* To put this possibility to the test we have been observing for some time now a depancreatized animal, not referred to in this article, fed with raw pancreas, as well as with meat and cane-sugar, and as yet (about three months) none of the symptoms described above have been observed.

These observations recall somewhat similar ones by Lombroso (1910), who found that whereas 80 per cent. of higher fatty acid was absorbed from the alimentary canal in animals in which no pancreatic juice was allowed to enter the gut, although a part or the whole of the pancreas remained in the body, very little was absorbed in completely depancreatized animals. To obtain the former conditions he either tied and cut the ducts of the pancreas, or transplanted the uncinate process as a graft under the skin of the abdomen

* It has therefore been suggested to us by A. R. Cushny that it may be because of the removal of this source of supply of lipase that in depancreatized dogs treated with insulin fat migration ultimately breaks down.

and removed the remainder of the gland. Lombroso suggested that the pancreas, perhaps indirectly through an internal secretion, has an influence on the absorptive function of the intestine. These conclusions have not, however, been uniformly confirmed by other observers. McClure, Vincent and Pratt (1916), for example, have failed to find that there is any difference in the efficiency of fat absorption in depancreatized dogs according to whether a portion of pancreas be left in the body or not.

It is certainly the case that absorption of fat and protein becomes extremely inefficient in depancreatized animals kept alive with insulin. Thus, we found that such animals could not tolerate any visible fat in their diet; it appeared in the fæcés in large amount and gastro-intestinal disturbance soon became marked. Neither could the animals tolerate polysaccharides, such as are contained in bread or biscuit, without developing diarrhœa. The markedly negative balance of nitrogen indicates also that protein was very imperfectly absorbed, and the possibility, suggested also by Dr. Almon Fletcher, must always be considered that the undigested protein in the intestine may, as a result of bacterial breakdown, produce toxic substances which by their action on the liver are responsible for the degenerative changes which have been described.

We are aware that much remains to be done in the investigation of the conditions described in this paper, but since it will take many months before this can be completed, we have deemed it advisable to place these observations on record. It requires no further evidence to establish the fact that although insulin is capable of removing the excess of fat which accumulates in the liver in depancreatized dogs, it cannot prevent an ultimate breakdown in the hepatic function, which leads to fatal symptoms and is associated with a very marked fatty infiltration of the organ.

We desire to express our thanks to Prof. O. Klotz for his valuable advice and criticism in connection with the microscopical portions of the work.

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[*Summary by the Editors.*—Although the administration of insulin leads to a disappearance of the fat which accumulates in the liver of dogs immediately after depancreatization, it is found that liver changes occur in depancreatized dogs kept alive for relatively long periods by the use of insulin. In cases where death followed, the liver contained very large amounts of fat (39·5 per cent.), which was deposited in large globules in the cells of the periphery of the lobules, while the cells at the centre of the lobules showed cellular degeneration with less fatty infiltration.

The absence of such changes in man in cases of diabetes which have been treated with insulin for longer periods suggests that the remaining pancreatic tissue plays some part in fat metabolism. Pending further investigations, some possible explanations are discussed.]

THE INFLUENCE OF MINERAL DEFICIENCY IN GOATS' MILK ON THE SKELETAL GROWTH OF KIDS.

S. V. TELFER AND J. A. CRICHTON.

From the Institute of Physiology, University of Glasgow, and the Rowett Institute for Research on Animal Nutrition, Aberdeen.

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SINCE the early work of Chossat in 1842, concerning the effects of a calcium-poor diet on the health and development of pigeons, a large number of observations have been made, which have served to indicate the importance of the mineral constituents of the diet on skeletal growth. That the development of the bones in young animals can be influenced very considerably by alterations in the mineral composition of their food has now been definitely proved. By means of feeding experiments on different species, it has been found that the range of variation through which the bones may be modified is not only wide, but that the amount of the bone-forming elements in the diet is one of the chief factors in determining the degree of ossification. In referring to their own experiments, Forbes and Keith (1914) remark: "It has been found possible greatly to decrease the central cavity in the long bones of pigs by feeding rations which are rich in bone-forming constituents. There seems to be no definite upper limit of phosphate deposit in the bones, as there is in the soft parts."

A gross deficiency of mineral matter in the diet gives rise invariably to osteoporosis, and apparently in certain groups of growing animals a rickets-like condition of the bones may become superadded. Elliot, Crichton and Orr (1922) have shown that rickets may be readily induced in young pigs by a diet deficient in bone-forming salts, and that the onset of the disease in these animals can be cured, and prevented, by the addition of a liberal amount of mineral constituents to the ration. In some recent experiments on dogs made by one of us (S. V. T.) rickets-like changes resulted in one group but osteoporosis alone in another, all the animals having the same calcium-deficient diet.

The work of McCollum, Simmonds, Shipley and Park (1921), and of Pappenheimer, McCann and Zucker (1922), has shown that the bone changes characteristic of rickets may be induced in rats on diets unbalanced in calcium and phosphorus.

In infants, by careful X-ray examination, the commencement of the bony changes may be recognized as early as the third month—a fact possibly of some significance in connection with dietetic factors in maternal milk and the causation of infantile rickets. It is of interest to note that several continental workers have published data tending to show that the milk of mothers of rachitic children is subnormal in lime. Bahrdt and Edelstein found 0·0426

per cent. CaO in normal breast-milk and 0·0315 per cent. CaO in the milk fed to rachitic infants. Schabad also noted that the calcium content of the milk of mothers of rachitic children was slightly below normal, but found that rickets could develop in infants on milk with the normal content of calcium. He therefore considered that a deficiency of calcium could not be the only cause of the disease.

Recently, Korenchevsky (1922) has referred to the possibility of a causal relationship between mineral deficiency in the diet and infantile rickets. During the past year an inquiry on the variations in food value and mineral content of the milk of nursing mothers has been conducted by one of us (S. V. T.), and is still in progress. The primary object was to ascertain if any appreciable deficiency of lime and phosphoric acid exists in the breast-milk of women suckling infants who have developed rickets. The observations so far made do not lend support to the view that such a deficiency does exist. In order to obtain some direct evidence of the effects of a mineral deficiency in the diet during the suckling period a further study was made. This consisted of a series of feeding experiments on kids carried out at the Rowett Institute, Aberdeen, the results of which are described in the present communication.

METHOD.

Eight animals, about the middle of April, 1923, when ten days old, were placed in pairs in separate pens on a concrete floor, each pair forming a single group. Sawdust was provided for litter.

The diets of the groups were as follows:

TABLE I.—*Diet of Animals per* 100 *c.c.*

Group 1.	Group 2.	Group 3.	Group 4.
100 parts fresh goats' milk	50 c.c. goats' milk 50 c.c. water 2·75 gm. lactalbumen 2·4 gm. lactose 3 c.c. butter-fat	25 c.c. goats' milk 75 c.c. water 4 gm. lactalbumen 3·5 gm. lactose 4 c.c. butter-fat	Same as Group 3 plus 2 gm. salt mixture.
$CaO = 0·21\%$ $P_2O_5 = 0·27\%$	$CaO = 0·18\%$ $P_2O_5 = 0·23\%$	$CaO = 0·17\%$ $P_2O_5 = 0·21\%$	$CaO = 0·36\%$. $P_2O_5 = 0·57\%$.

The animals were fed quantitatively four times daily from bottles provided with teats. Considerable care was exercised in the reconstitution of the experimental diets of Groups 2, 3 and 4. The fat, lactose and lactalbumen employed for this purpose were accurately weighed, and the feeding throughout the experimental period was conducted personally by one of us (J. C.). The percentages of protein, carbohydrate and fat for all the diets were nearly equal. At first the fat of goats' milk was used in partly reconstituting the diets of the experimental groups, but later butter-fat had to be employed. Though the lactose was nearly pure, the lactalbumen contained a considerable amount of earthy salts for which allowance had to be made. The alterations in the mineral content of the food of the experimental groups were effected in the following manner:

Fresh goats' milk, the sole diet of the control animals of Group 1, was diluted one-half for the diet of Group 2 and three-quarters for that of Group 3, all the mineral constituents of the milk being reduced in proportion to the added water. The reconstitution of the protein, carbohydrate and fat of these diluted milks necessitated the introduction of mineral matter in appreciable quantity, the lactalbumen available being the chief source of this supply. The mineral content of the diet of Group 2 when finally adjusted was found on analysis to be less than that of the normal diet, and a still greater reduction had been effected in the diet of Group 3 (*vide* Table I). In Group 4 the diet was the same as Group 3, except for the addition of an excess of minerals in the form of a salt mixture roughly corresponding in percentage composition to the ash of goats' milk and made up as follows: calcium lactate 0·8 gm., acid potassium phosphate 0·8 gm., ferric citrate, ·004 gm., magnesium sulphate 0·07 gm., sodium chloride 0·15 gm. Two grams of this mixture were added to each 100 c.c. of the diet of Group 4.

It will be observed that the dilution of the natural milk, by which the reduction of mineral matter was effected in the diets of the experimental groups, was considerable. In Group 3, for example, only one-quarter of the diet was derived from natural sources. The lactose, butter-fat and lactalbumen used in reconstituting the milks of Groups 2, 3 and 4 could not rigidly be regarded as supplying the same quality of carbohydrate, fat and protein as exists in goats' milk, though the amounts in the diets were the same as those in Group 1. Any organic factors specific to goats' milk and essential for the normal metabolism and growth of kids were present in the diets of Groups 3 and 4 in only one-fourth of the concentration of the natural diet of Group 1, as a result of the dilution.

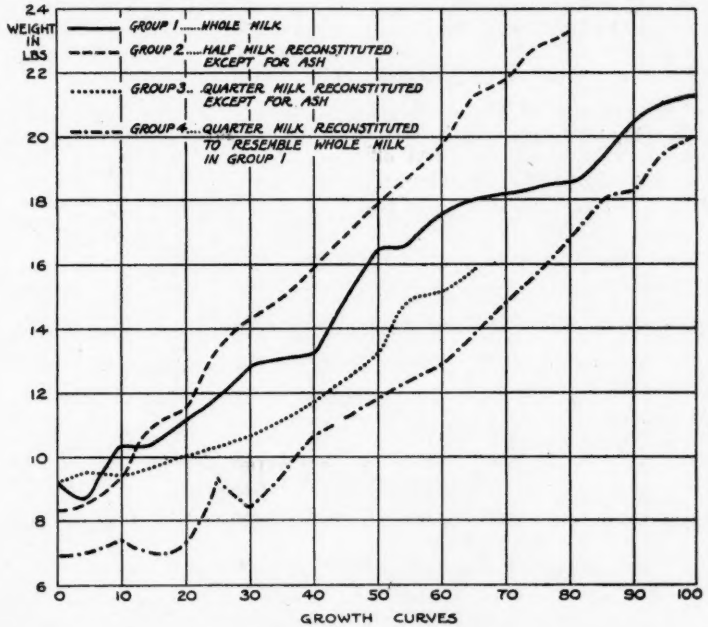
From time to time the rations were increased as the animals gained in weight. When they were two months old the retentions of lime and phosphoric acid were determined by balance experiments. Each animal was placed in a metabolic cage and the urine and fæces collected separately and quantitatively over a period of 4 days. The estimations of lime and phosphoric acid in the excreta were made by the usual gravimetric methods. From these results the total amount of lime and phosphoric acid eliminated by each animal was determined, and by deduction from the intake during the experimental period the retentions of each per kilo of body-weight per day were calculated (*vide* Tables II and III).

Finally, on July 16th the male member of each group was killed by bleeding under chloroform anæsthesia, and the long bones dissected out for examination and comparison. In each case the left femur was cleared of all soft tissue, split longitudinally, and the marrow, epiphysial lines and shaft thicknesses inspected. The split specimens were placed in a mixture of ether and alcohol for one month after careful removal of the marrow from the shafts, then dried in a water oven and weighed. The mineral matter in each specimen was estimated by ashing the dried bones in a muffle furnace. (*vide* Table IV). 100 c.c. blood were also drawn from the carotid artery, and the total calcium of the separated serum estimated in each specimen (*vide* Table V).

RESULTS.

1. *Growth.*

The appetites of the animals in Groups 1 and 4 were excellent throughout. When three weeks old the condition of the kids in Groups 2 and 3 tended to deteriorate, but there were no objective signs of ill-health. All four animals left a little of their feeds at this time, and some coaxing was necessary to induce them to swallow the last few c.cs. of their rations. After a short interval the animals in Group 2 were as easily fed as those on the natural milk (Group 1).



Those in Group 3 never regained a normal condition. Their appetites lessened, and though they continued to gain in weight, the rate of increase was less than normal (*vide* growth curves), and there was a progressive deterioration of their physical state. Lessened activity was at first evident, and gradually this became a feature in both animals. While the others were running about in their pens or trying to clamber over the wooden sides they remained abnormally quiet and listless, and were often found lying down in a corner. In this manner their behaviour was in striking contrast to that of their vigorous neighbours. They also resented being stroked and petted. They finally presented a stunted appearance.

When six weeks old it was evident that both these animals had almost ceased to grow. Distension of the stomach and delayed emptying of the

contents then became apparent. Succussion could be easily elicited several hours after a feed. This sign was not manifest in the animals of the other groups till a much later date. Occasionally diarrhœa was noticed.

On June 20th one of the animals of Group 3 died. A post-mortem examination was conducted by Dr. McGowan, of the Rowett Institute. The stomach was filled with fluid and contained some sawdust. Petechial hæmorrhages were seen in the mucosa. The kidneys were swollen and hæmorrhagic and albumen was found in the urine. No abnormal blood changes were evident.

The two animals in Group 4, fed on the same diet as those of Group 3 but with the addition of the salt mixture, remained in excellent condition throughout the experiment.

2. *Metabolic Experiments.*

The distribution of lime and phosphoric acid in the urine and fæces is shown in Table II; the results of the balance experiments are recorded in Table III.

TABLE II.—*Distribution of CaO and P_2O_5 in Excreta* (4 *days*).

A. *Urine.*

	Group 1.		Group 2.		Group 3.		Group 4.	
	No. 1.	No. 2.	No. 3.	No. 4.	No. 5.	No. 6.	No. 7.	No. 8.
Amount excreted, c.c.	3090	2870	3039	2867	Animal died on June 20th.	2235	2690	2548
CaO, gm.	0·16	0·11	0·16	0·14		0·11	0·19	0·16
P_2O_5, gm.	4·86	4·91	4·52	4·62		3·98	15·17	12·62

B. *Fæces.*

Amount excreted (dry), gm.	21·5	25·4	29·6	33·5	56·5	64·6	40·2
CaO	4·568	3·378	3·61	2·91	3·27	5·16	3·61
P_2O_5	2·064	1·39	1·51	1·17	1·29	2·01	2·31
Total mineral matter, per cent.	36·1	25·6	23·8	19·25	12·2	18·4	19·8

TABLE III.—*Retention of CaO and P_2O_5* (4 *days*).

	Group 1.		Group 2.		Group 3.		Group 4.	
	No. 1.	No. 2.	No. 3.	No. 4.	No. 5.	No. 6.	No. 7.	No. 8.
Weight (kilos)	7·7	5·7	7·5	5·7	—	5·2	7·8	6·6
CaO { intake	9·02	7·38	9·84	9·6	—	6·57	15·30	12·42
CaO { output	4·72	3·48	3·77	3·05	—	3·38	5·30	3·77
Retention, 4 days	4·30	3·90	6·07	6·55	—	3·19	10·05	8·65
Retention per kilo per day	0·14	0·17	0·20	0·26	—	0·15	0·31	0·32
P_2O_5 { intake	11·88	9·72	11·07	10·8	—	7·01	24·22	19·66
P_2O_5 { output	6·82	6·30	6·03	5·79	—	5·27	17·18	14·93
Retention, 4 days	5·06	3·42	5·04	5·01	—	1·74	7·04	4·73
„ per kilo per day	0·16	0·15	0·17	0·21	—	0·08	0·22	0·18

The amount of calcium excreted in the urine was small, nearly all having been eliminated in the fæces. The urinary phosphorus was much greater than the fæcal phosphorus in all the groups; the larger urinary elimination of this

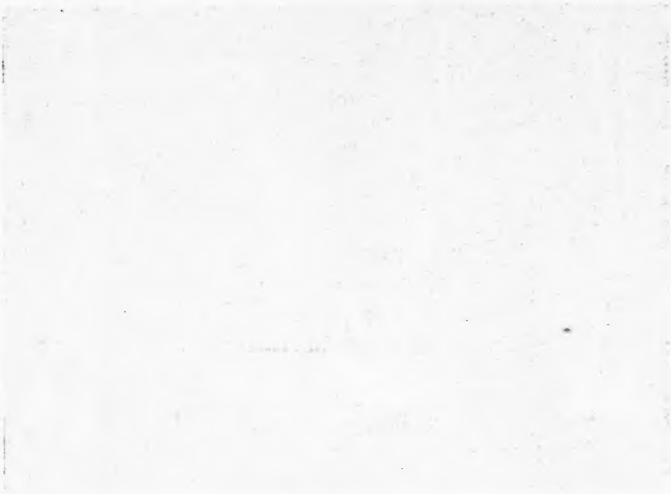


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FIG. 1.

FIG. 2.

Telfer and Crichton.

element in Group 4 naturally followed the larger intake. It is of interest to note that in the adult animal phosphoric acid is excreted almost entirely by the bowel.

There was a positive retention of both elements in all the animals, though the retention of phosphoric acid was evidently abnormally low in the surviving animal belonging to Group 3. The retentions were greatest in the animals of Group 4, in which the intakes of lime and phosphoric acid were highest. The period of the metabolic experiments was too short, however, to permit of accurate comparison of these retention values in the different groups.

3. *Examination of Bones.*

No signs of rickets were evident in any of the specimens. The lower epiphyses of the radius and ulna in each case were normal in thickness and outline. Striking differences were apparent in the density and thickness of the compact bone of the femoral shafts. The cut margins of the left radial shaft taken from the male member of each group are represented in the Plate (fig. 1). The specimen from Group 3 is thin and fragile, but a distinct gradation in thickness is noticeable on passing from Group 1 to Group 3. The specimen from Group 4 is the densest of all. The animals in this group had throughout a much higher intake of minerals than those of Group 1, and, as has been seen, the retention of the bone-forming elements per kilo of body-weight was also highest in Group 4. For purposes of comparison the lower end of the left femur from each male animal in Groups 3 and 4 is shown in fig. 2. The diets of these two groups were identical in every respect except that of mineral content.

TABLE IV.—*Mineral Matter of Femur.*

	Group 1.	Group 2.	Group 3.	Group 4.
Weight of dried femur	29·236	28·84	19·04	26·59
Femoral weight per kilo	1·39	1·20	1·19	1·47
Mineral matter of femur	41·8 %	41·5 %	37·2 %	42·0 %
Ditto. per kilo body-weight	0·583	0·499	0·443	0·62

The results recorded in Table V corroborate the findings obtained on inspection. The weight of the femur per kilo of body-weight in Groups 2 and 3 (Ca-deficiency diets) was less than that of the animals fed on fresh undiluted milk (Group 1); but the animals fed on the mineral excess (Group 4) had the greatest amount of mineral matter both relatively and absolutely in their bones. This result is noteworthy. In spite of the differences in the rate of calcium deposition which must have been in progress, and, in one of the groups, gross impairment of nutrition as well as faulty skeletal growth, the calcium content of the blood-serum was practically the same in all.

TABLE V.—*CaO Content of Serum.*

	Group 1.	Group 2.	Group 3.	Group 4.
CaO mgm. per 100 c.c.	14·3	14·9	14·7	14·7

DISCUSSION.

The results of these experiments afford still further evidence of the great importance of an adequate mineral intake for normal metabolism and growth during early life. The reductions in the mineral content of the diet were followed by defective growth, not only of the bones, but of the other tissues of the body. The effects produced by the smaller reduction were shown only in the lessened deposition of bone-salts (Group 2). A further diminution in the mineral intake led to profound disturbance of metabolism as well as defective ossification (Group 3).

The mineral content of the milk of different mammals bears a relation to the rate of growth of the young during the early period of life, and the concentration of mineral matter as well as its composition must be regarded as adapted to the growth and development of the young of any given species. An alteration in the natural intake of salts during the period of suckling might therefore be expected to lead to some changes in metabolic activity. The results show that these changes can be effected by comparatively small reductions. It might be argued that, in the process of modifying the milk, extensive alterations had been made in the natural diet in addition to the reduction of mineral matter, and that these, whatever they might be, were responsible for the effects produced and not the lack of minerals. Since the diet of the animals in Group 4 was identical with that of Group 3 in every respect except that of mineral matter, it must be concluded that the results obtained were due to deficiency of mineral matter alone. The animals on the excess of minerals not only continued to grow in a perfectly healthy manner, but had thicker and heavier bones than those fed on the natural unmodified milk (Group 1). It would appear that the mineral content of the milk could not be diminished to any appreciable degree below the normal without risk, firstly of lessened deposition of bone-salts, and secondly, impaired nutrition and disordered metabolism.

These results also corroborate the findings of many previous observers who had studied the effects of variations in the mineral content of the rations on the growth of bone in farm animals. They are of interest in demonstrating that the skeletal development of sucklings can be affected in exactly the same manner by modifying the maternal milk. In the present defective state of our knowledge of mineral metabolism it is impossible to do more than speculate on the origin and course of the abnormal processes which follow an insufficient utilization of minerals. That an extremely important *rôle* must be played by the inorganic elements of the food during the period of active growth in early life can be readily understood. There is then a rapid utilization of calcium and phosphorus for bone development, together with an increasing need of salts to preserve the ionic concentration of the body-fluids.

In an adult animal, or in one beyond the early period of active growth when bone formation is somewhat advanced, a deficiency of mineral matter in the diet will have less immediate effect on nutrition, since a store which may to some extent be drawn upon to maintain the salt equilibrium of the body-fluids is then in existence, and the rate of increase of growth has diminished. The depletion of the bones of calcium during pregnancy and lactation,

especially on a Ca-poor diet, is a normal physiological occurrence, and the loss of a considerable amount of mineral matter from the bones in certain diseases may take place without alteration of the normal concentration of calcium in the soft tissues and blood. It has also been shown by Findlay, Noël Paton and Sharpe (1921) in the human subject that there is a steady diminution in the positive retention of calcium with age.

During the earliest period of life, when growth is most active, the store of mineral elements, with the possible exception of iron, is relatively small, and the essential requirements for tissue increase, and especially for the rapid extension of the skeletal system, must be derived wholly from the diet. A high positive retention of the elements, and particularly of calcium and phosphorus, being then required, a small deficiency of these elements may be of grave importance. E. V. McCollum (1922), discussing disturbances of growth, refers to calcium as one of the chief of the dietary factors of significance in health. He also states: "There is much evidence that the calcium intake of breast-fed infants is as a rule not far from the minimum on which normal growth can take place."

Although further study is necessary before any attempt can be made to formulate exactly the dietetic conditions for normal growth, these experiments seem to indicate that whatever organic factors may be essential, profound nutritional disturbances are consequent on comparatively trivial alterations of the inorganic constituents of the diet during early life.

SUMMARY.

Artificial feeding experiments with goats' milk modified in mineral content were carried out from birth on kids with the following results:

(1) Control animals fed on the unaltered milk developed normally.

(2) A very slight reduction of the mineral content induced a perceptible degree of osteoporosis, but otherwise the animals remained in good condition and increased normally in weight.

(3) A slight further reduction was followed, after an initial period of growth, by definite signs of impaired nutrition, of which the more prominent were—falling off of rate of growth, and finally stunting, loss of activity, and markedly defective ossification. The death of one animal ensued.

(4) The addition of an excess of a salt mixture, roughly similar to the ash of goats' milk in composition, entirely obviated these pathological changes. The animals developed normally, and the degree of ossification of the limb bones was greater than than of the animals fed on unmodified milk.

(5) From these results it is suggested that—

(*a*) A comparatively small deficiency of mineral matter in the diet of a suckling animal may give rise to severe nutritional disturbance with defective deposition of bone-salts.

(*b*) The normal mineral content of milk is probably near the minimal requirements of the species, since the rate of ossification can be considerably increased by a greater concentration of mineral matter, and a small reduction is followed by defective growth and impaired nutrition.

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THE EXPERIMENTAL PRODUCTION OF BILIARY CIRRHOSIS BY SALTS OF MANGANESE.

G. MARSHALL FINDLAY.

From the Laboratory of the Imperial Cancer Research Fund, London.

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CHRONIC manganese poisoning among industrial workers has now been recorded on the Continent, in America, and more recently in this country by Charles (1922). The clinical symptoms, which are not unlike those associated with progressive lenticular degeneration, have been fully described, though little is yet known in regard to the pathological changes occurring in this form of poisoning. So far Casamajor (1916) has described the only case in man where a pathological examination has been made after death. In addition to lesions in the medulla biliary cirrhosis was present.

Attempts to produce chronic manganese poisoning in animals have not been very successful. Reiman and Minot (1920) fed dogs on rhodenite (manganese metasilicate) for more than a year without producing any pathological changes in the tissues, while Drinker, Shaw and Drinker (1923) found that cats showed fibrosis of the liver after receiving a single massive dose of manganese, though no pathological details are given.

Numerous experiments, however, have been carried out on the fate of single doses of manganese injected into animals. Thus Lund, Shaw and

Drinker (1923) showed that manganese dioxide and manganese metasilicate have little permanence as foreign substances in the body; they are arrested in the liver to the practical exclusion of other organs, and are thence excreted in the bile, largely in the form of a soluble manganese carbonate. When large doses are administered parenterally a very small amount of manganese may appear in the urine.

In view of the absence of precise knowledge in regard to the toxic effects of manganese it seemed to be of interest to investigate the histological changes produced in animals by salts of this substance. For this purpose the soluble manganous chloride was employed.

I. EXPERIMENTS ON RABBITS.

A. *The Effect of the Parenteral Administration of Large Doses.*

Subcutaneous injections of a solution of manganous chloride in water were given to ten rabbits with the usual aseptic precautions. The actual dose of manganous chloride varied from 10 to 60 mg. In every case death took place in from twenty-two to forty-eight hours after the injection. Manganous chloride is thus extremely toxic to rabbits, as is shown by Table I:

TABLE I.

	Weight of rabbit in gm.	Doses in mg.	Period of survival in hours.
1	1800	10	48
2	1450	15	40
3	1400	20	48
4	1550	25	36
5	1500	35	40
6	1250	40	42
7	1750	45	28
8	1450	50	22
9	1400	55	24
10	1850	60	26

A few hours after the injection the animals became quiet and listless. They refused food, gradually became weaker, and eventually passed into a semi-comatose state. The urine after death was loaded with albumin, together with red blood-cells, casts, and a trace of bile. On post-mortem examination the appearances were closely similar in all cases. The liver was always slightly enlarged, friable, and of a pale yellow colour, often with reddish mottlings. The spleen varied considerably in size, but as a rule was enlarged and of a deep red colour. The kidneys were pale, the lungs and intestines congested. Microscopically the liver showed degeneration of the parenchyma, amounting in some areas to definite necrosis. Sections stained with Sudan III showed the presence of fatty changes, especially at the periphery of the lobules. A few of the Kupfer cells were swollen and prominent, but there was no undue proliferation of the endothelial cells. In some sections small hæmorrhagic foci could be noted scattered throughout the liver substance. In the kidneys the main changes were found in the glomeruli.

Many of the tufts were congested, and in several there was blood in the capsular space. The effect on the tubular cells was unequal. In some cases they were apparently normal, while in others there was fatty change. In the spleen there was congestion of the sinuses; the other organs showed no constant histological changes.

B. *The Effect of Repeated Subcutaneous Injections.*

Preliminary experiments had shown that after the subcutaneous injection of 5 mg., rabbits, although obviously unwell for a few hours, finally recovered completely.

Two series of young rabbits were therefore inoculated subcutaneously with solutions of manganous chloride. The first series received 3 mg., the second series 5 mg. every second day. The main pathological effects, apart from the occasional occurrence of local necrosis at the site of injection, were a new formation of connective tissue in the liver and proliferation of the epithelium lining the bile-ducts.

1. *Experiments in which 3 mg. were given every second day.*—Ten rabbits were used in this experiment. The first rabbit was killed ten days after the beginning of the experiment, the others at intervals up to the sixtieth day of the experiment. For some hours after each injection the animals were quiet and refused food. As a rule they increased only very slightly in body-weight during the course of the experiment—in many cases in fact they showed an actual decrease.

All the animals were free from coccidiosis, and in only two cases were there tape-worm cysts in the mesentery. At post-mortem examination the animals were found to be rather poorly nourished. There was occasionally an icteric tint in the subcutaneous tissues, but no evidence of ascites. The liver was slightly enlarged, smooth and rather friable, deep red in colour or with red and yellow mottlings. There was no thickening of the capsule and no pathological adhesion to surrounding structures. The spleen was normal in size, but of a deep purple-red colour. The kidneys were pale but of average size. The lungs, heart, pancreas, suprarenals and intestine showed no naked-eye changes. On microscopical examination the liver showed some fatty degeneration restricted chiefly to the periphery of the lobules. The extent of the fatty change appeared to vary with the period which had elapsed since the time of the last injection and the death of the animal, being more extensive when the animal was killed a few hours after an injection. The vessels in the portal tracts were congested. The capsule was not thickened. In the portal tracts, however, there were masses of small round-cells, together with the formation of new fibrous tissue. Numerous new bile-capillaries were present and each of these twigs was surrounded by freshly-formed fibrous tissue. Many of the larger bile-ducts were dilated, while the epithelial cells lining the ducts showed numerous mitotic figures. In some animals the bile-duct epithelium was thrown into folds, the appearance being similar to that described by Vaughan Harley and Barratt (1896) as having been produced in cats by the experimental obstruction of the hepatic ducts.

In two rabbits the bile-duct epithelium had taken on a papillomatous

appearance. Although very careful search was made in these two animals for evidence of coccidial infection, none could be found. The amount of round-cell infiltration and fresh fibrous-tissue formation was much greater in the livers of those animals which had received five or more injections. The splenic sinuses were engorged with red cells, but there was no increase of fibrous tissue. The kidneys exhibited only some slight fatty change in the tubular cells.

2. *Experiments in which 5 mg. were injected subcutaneously every second day.*—The rabbits were very unwell for about twenty-four hours after the injections. Of the rabbits used in this experiment one was found to be suffering from coccidiosis and was therefore discarded. Particulars of the rabbits are shown in Table II:

TABLE II.

	Weight in gm. at beginning of experiment.	Weight in gm. at end of experiment.	Date of death in days.
31	1520	1380	17
32	1440	1400	17
33	1100	1020	21
34	1220	1080	22
35	1450	1300	23
36	1540	1200	25
37	1400	1350	Suffering from coccidiosis.
38	1420	1400	31
39	1420	1280	35
40	1500	1200	35
41	1630	1640	21
42	1700	1480	26
43	1750	1730	38
44	1560	1430	58
45	1420	1400	2
46	1950	1980	45
47	1870	1920	Still alive.
48	1230	1200	42
49	1380	1420	35
50	1180	970	50
51	1310	1260	50
52	1150	1050	39

In the case of rabbits Nos. 31–40 the experiments were carried out in the Research Laboratory of the Royal College of Physicians of Edinburgh, the later experiments in London. Control rabbits, examined both in Scotland and in London, failed to show pathological changes in the internal organs similar to those exhibited by the experimental animals. With two exceptions all the rabbits underwent a progressive diminution in weight as the result of the injections. The urine contained albumin, casts and sometimes a trace of bile. Rabbits Nos. 44 and 50 showed rigidity of the hind limbs a few days before death.

On post-mortem examination there was no evidence of ascites though jaundice was sometimes present. The liver was enlarged, soft and friable. It was either deep red or reddish-yellow in colour. The gall-bladder was not unduly distended. The capsule was not thickened, nor was there any abnormal adhesion to surrounding structures.

The spleen did not show any definite enlargement; it was purplish-red in colour. The kidneys were normal in size but pale. The other organs presented no naked-eye lesions.

Microscopically the changes were very similar in every case with the exception of rabbit No. 45, which only showed diffuse fatty change in the liver. In all the others the lesions in the liver varied in degree rather than in kind. The liver-cells, except those immediately round the central vein, stained poorly. At the periphery of the lobules, in some animals, many of the hepatic cells were shrunken and atrophied while the intralobular capillaries were greatly congested. The Kupfer cells were not enlarged. In the portal spaces there was an excess of small round-cells together with freshly formed fibrous tissue, strands of which divided up the liver substance into areas which for the most part corresponded with pre-formed lobules. The bile capillaries were proliferated, each bile-duct being surrounded by newly formed connective tissue.

In the spleen the sinuses were packed with red blood-corpuscles, while many of the endothelial cells contained much blood-pigment. There was no obvious formation of fresh fibrous tissue. In the kidneys the glomeruli were congested and in many cases there was hæmorrhage into Bowman's capsule. The intertubular vessels were deeply congested. The tubular cells showed fatty changes somewhat irregularly distributed throughout the section. The lumina of many of the tubules were filled with casts, but only a very slight formation of fresh fibrous tissue could be detected in a few animals.

Rabbit No. 50 is of interest, for during the course of the experiment it became pregnant and gave birth to five apparently normal young. Two of these were killed at birth and two when fifteen days old. The livers of all were quite healthy. The mother had cirrhosis.

The main change produced in rabbits by the subcutaneous injection of manganous chloride is thus seen to be a form of liver cirrhosis of the biliary type.

Certain objections have, however, been urged against the use of rabbits for work on cirrhosis. Thus Ophüls (1908) has stated that owing to the frequency of coccidiosis in the livers of rabbits, all cases of cirrhosis in these animals must be looked upon with suspicion. This objection has been combated by Gye and Purdy (1922), who, in their work on the effects of colloidal silica on the rabbit, point out that the lesions produced by coccidiosis are circumscribed, and that even when the liver is infected there is little difficulty in fixing their limit. In only one animal in the present series was there any evidence of coccidiosis.

A more serious objection has, however, been made by Wells (1920), who finds that the repeated injection in rabbits of almost any foreign protein material, such as Witte's peptone, foreign blood or emulsions of organs may be followed by the occurrence of cirrhotic changes in the liver. "Peptone" injections in dogs and guinea-pigs failed to cause a similar cirrhosis.

Rabbits would thus seem to be especially liable to develop cirrhotic changes in the liver under comparatively slight provocation. Before drawing any definite conclusion as to the effects of manganous chloride on the liver, it therefore seemed desirable to investigate the effects of this salt on other laboratory animals. Experiments were accordingly carried out on guinea-pigs and rats.

II. EXPERIMENTS ON GUINEA-PIGS.

Preliminary experiments had shown that in proportion to their body-weight guinea-pigs are relatively less susceptible than rabbits to the toxic action of manganous chloride.

Six guinea-pigs were given subcutaneous injections of 3 mg. of manganous chloride dissolved in distilled water every other day. Particulars of the animals are given in Table III:

TABLE III.

	Weight of animal in gm. at beginning of experiment.	Weight of animal in gm. at end of experiment.	Period of survival in days.
1	285	220	12
2	380	350	29
3	300	310	32
4	290	225	34
5	270	270	39
6	260	270	44

Guinea-pig No. 1 died from an intercurrent broncho-pneumonia. The liver showed slight fatty change, but no increase in fibrous tissue.

All the other animals showed some degree of fibrosis of the liver, the change being most pronounced in guinea-pig No. 5. In this animal the liver was reduced in size and showed red and yellow mottlings. The other organs were macroscopically normal. Microscopically in the liver there were seen to be patches where the parenchyma cells were replaced by masses of loose connective tissue of a somewhat less cellular character than that seen in the rabbit. The fibrosis was well seen in the region of the portal tracts and was associated with proliferation of the smaller bile-ducts. The liver parenchyma had undergone fatty degeneration. There was no enlargement or proliferation of the Kupfer cells. The microscopical appearances of the liver are shown in Fig. 4, from which it will be seen that the type of lesion produced is similar to that met with in the rabbit. The changes met with in livers of the other guinea-pigs varied only in degree from that illustrated in Fig. 4. Control guinea-pigs kept under similar conditions and receiving the same food showed no fibrosis of the liver. A slight degree of proliferation of the bile-duct epithelium was seen in two of the animals examined, but it was never so definite as in the rabbit.

III. EXPERIMENTS ON RATS.

A few experiments were also carried out on rats. Ten rats were given 3 mg. of manganous chloride by subcutaneous injection every other day.

Unfortunately subcutaneous injections of manganous chloride in the rat are very liable to produce local necrosis. In the first four weeks of the experiments four of the animals died from septic infection. Neither macro- nor microscopically were there any characteristic lesions in these rats. In the remaining six animals there was present in all more or less fibrosis of the liver. In rats 9 and 10 there was noticed a certain rigidity of the hind legs and head, though choreiform movements were not seen at any time. Particulars of the animals are given below:

Rat 2 died on the 38th day of the experiment. Weight 92 gm.—a decrease of 28 gm. The liver was enlarged and yellow in colour, with some reddish mottling. There was jaundice, but no ascites. The spleen was greatly enlarged, weighing 1·19 gm. Microscopically the liver showed a very slight increase in fibrous tissue, which was almost entirely restricted to the region of the portal tracts. There was very little round-celled infiltration. The other organs were normal, with the exception of the spleen, which showed congestion.

Rat 3 died on the 42nd day of the experiment. Weight 63 gm.—a loss of 31 gm. The liver was yellowish-white in colour, the spleen slightly enlarged. Microscopically the changes in the liver, illustrated in Fig. 5, were closely similar to those in rat 2.

Rat 4 died on the 49th day of the experiment. Weight 72 gm.—a loss of 25 gm. There was jaundice but no ascites. The liver was enlarged and yellow in colour. The other organs appeared normal except for broncho-pneumonic patches in the right lung.

Microscopically there was evidence of a well-marked perilobular fibrosis, which divided the liver up into monolobular areas. The connective tissue was loose in structure and was associated with a considerable degree of round-celled infiltration. There was also a slight degree of proliferation of the bile-capillaries, as is seen in Fig. 6.

Rat 7 died on the 53rd day of the experiment. Weight 82 gm.—a loss of 31 gm. Both the naked-eye and microscopic appearances were very similar to those in rat 4.

Rat 9, killed when moribund on the 84th day of the experiment. Weight 58 gm.—a decrease of 37 gm. The animal was emaciated. There was no œdema or ascites, but jaundice was present. The liver was normal in size, firm, and of a lemon-yellow colour. The outer surface was slightly roughened. There were no pathological adhesions. The spleen, which was a little enlarged, was of a deep red colour. The right lung showed patches of broncho-pneumonia. The other organs appeared normal. Microscopically the liver showed an intense fibrosis, not confined to the portal regions, but extending into the lobules and dividing up the liver-cells into small irregular areas. The fibrous tissue contained a number of small round-cells. In some parts of the liver the fibrous tissue was so dense that practically no liver-cells remained. In other parts a few liver-cells were noticeable, many of them of a hypertrophic character with giant nuclei. All the surviving liver-cells showed fatty degeneration. The other organs did not show any abnormal changes.

Rat 10 killed on the 86th day of the experiment, moribund. Weight 86 gm.—a loss of 24 gm. The liver was normal in size. It was of a yellow

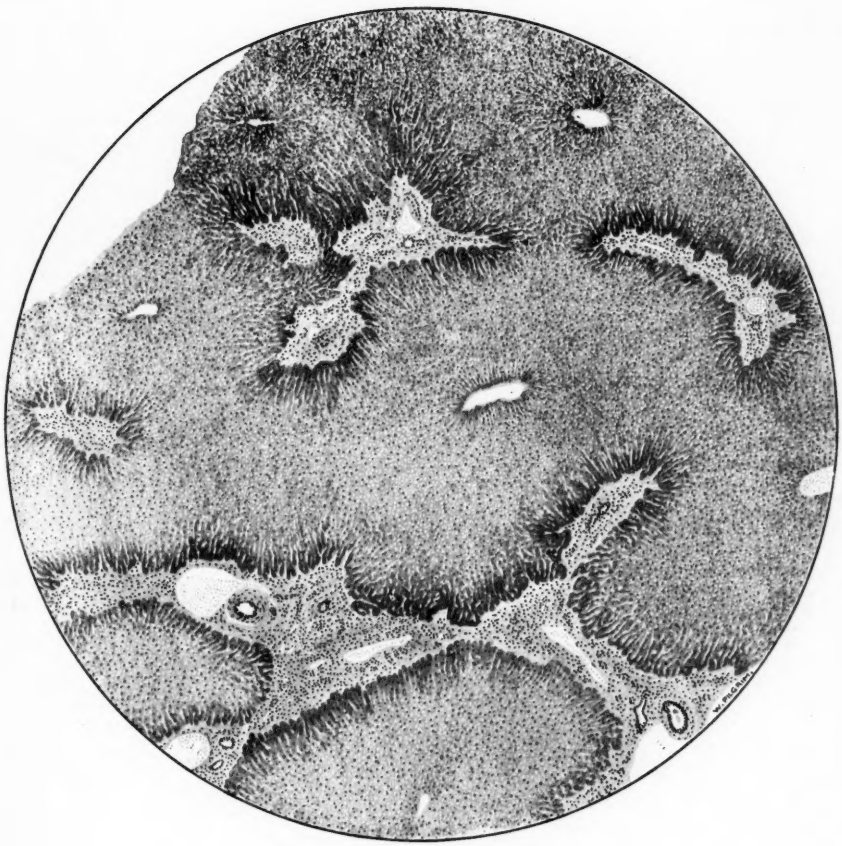

FIG. 1. Scale 56 : 1.

Stained iron-hæmatoxylin and eosin.

Liver of rabbit 35, which received subcutaneous injections of 5 mg. of manganous chloride for 5 weeks. The fibrosis is closely related to the portal spaces and is accompanied by round-celled infiltration and a formation of fresh bile-capillaries.

Findlay.

FIG. 2. Scale 260 : 1.

Stained iron-hæmatoxylin and eosin.

Liver of rabbit 51, which received subcutaneous injections of 5 mg. of manganous chloride for 9 weeks. The epithelium of the bile-duct forms papillary processes.

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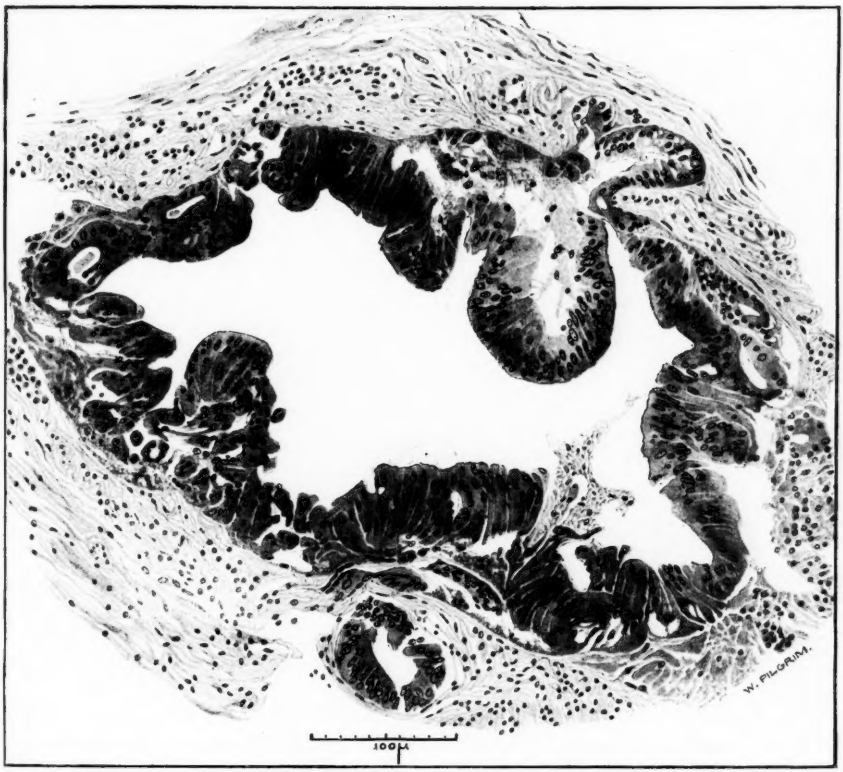

FIG. 3. Scale 300 : 1.

Stained iron-hæmatoxylin and van Gieson.

Liver of rabbit 46, which received subcutaneous injections of 5 mg. of manganous chloride for 6 weeks. Note the formation of new bile-capillaries and the proliferation of the epithelium of the larger bile-duct.

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FIG. 4. Scale 110 : 1.

Stained iron-hæmatoxylin and van Gieson.

Liver of guinea-pig 5, which received subcutaneous injections of 3 mg. of manganous chloride for 3½ weeks. The freshly formed fibrous tissue is most marked in the portal spaces; it should be compared with that shown in Fig. 1.

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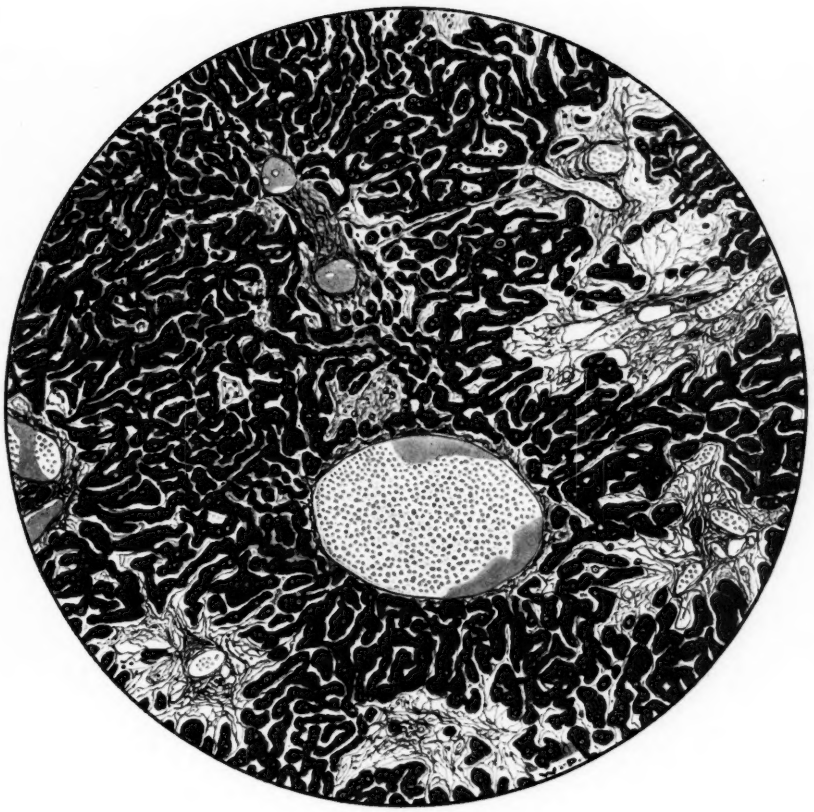

FIG. 5. Scale 130 : 1.

Stained iron-hæmatoxylin and van Gieson.

Liver of rat 3, which had received subcutaneous injections of 3 mg. of manganous chloride for 4¾ weeks. Note the early formation of fresh fibrous tissue in relation to the portal spaces.

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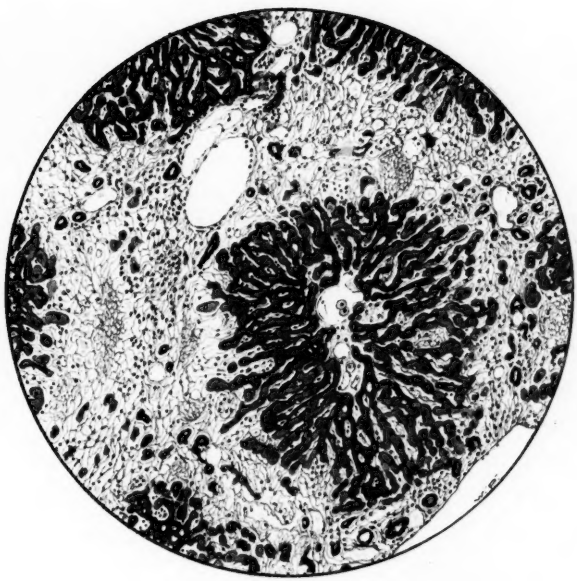

FIG. 6. Scale 90 : 1.

Stained iron-hæmatoxylin and van Gieson.

Liver of rat 4, which had received subcutaneous injections of 3 mg. of manganous chloride for 7 weeks. The freshly formed fibrous tissue forms a complete ring round the periphery of the lobules.

Findlay.

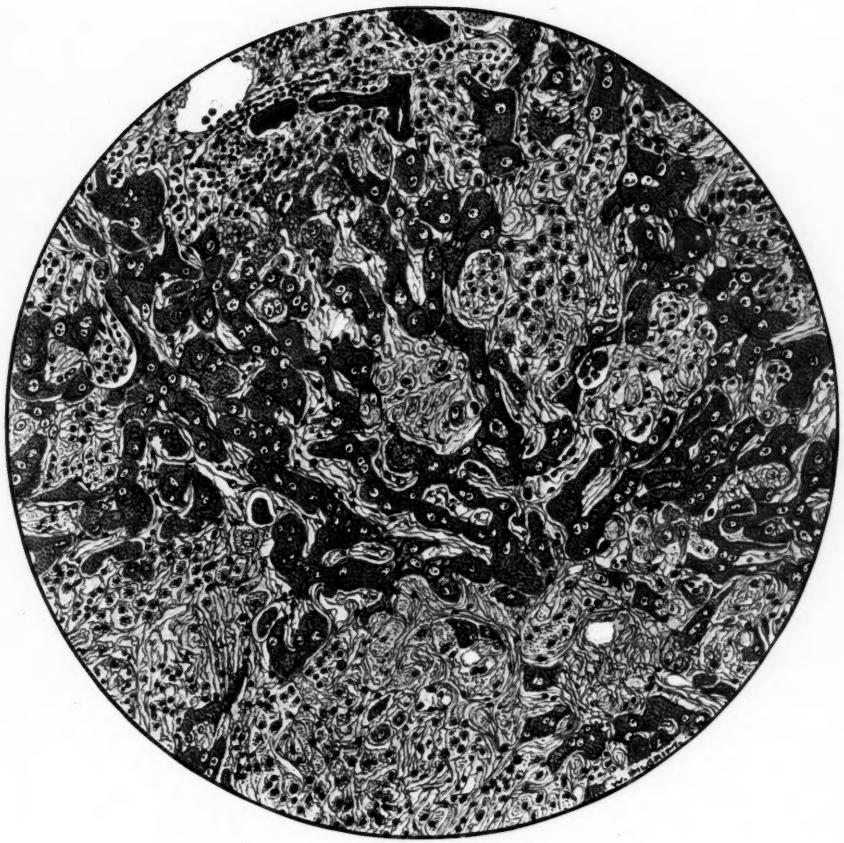

FIG. 7. Scale 160 : 1.

Stained iron-hæmatoxylin and van Gieson.

Liver of rat 9, which had received subcutaneous injections of 3 mg. of manganous chloride for 12 weeks. The freshly formed fibrous tissue has invaded the lobule. Note the hypertrophic liver-cells.

Findlay.

UNIV. OF MICH.

colour with reddish mottling. The other organs appeared macroscopically normal. Microscopically, in the liver there was a moderate degree of cirrhosis confined to the periphery of the lobules, similar to that described in rat 4.

An attempt was also made to produce a similar cirrhotic condition in rats by feeding them on manganous chloride.

Twelve rats were given from 0·3–0·4 gm. of manganous chloride per day in addition to their ordinary diet. The animals remained in good condition, and in many cases continued to increase in weight during the course of the experiment. Death usually took place after from six to ten weeks, and in every case a certain degree of cirrhosis of the liver was found. The histological appearances were entirely similar to those described in the rats which had been injected with manganous chloride. In three of the rats fed on manganous chloride there was rigidity of the limbs for some days before death, while in all jaundice was noted at the post-mortem.

The livers of a number of control rats kept under similar conditions were entirely free from fibrosis.

CONCLUSIONS.

When injected subcutaneously in large doses manganous chloride is extremely toxic to the parenchyma of the liver and kidney. In small repeated doses its toxic action is chiefly exerted on the liver. It has been possible to produce by repeated subcutaneous injections of manganous chloride an experimental form of liver cirrhosis in rabbits, rats and guinea-pigs, while a similar condition has also been brought about in rats by the oral administration of the same salt. In its earliest stages the fibrous overgrowth in the liver is confined to the periphery of the lobules in relation to the portal spaces, the type of cirrhosis being very definitely monolobular. In its later stages the freshly formed fibrous tissue invades the whole of the lobules. Jaundice is often present. A possible clue to this action of manganous chloride may be found in the fact that manganese salts are very largely excreted from the body by means of the bile.

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STANDARDIZATION OF ANTI-DYSENTERY (SHIGA) SERUM.*

H. J. SUDMERSEN, PH.D., MISS B. F. RUNGE, B.SC., AND R. A. O'BRIEN, M.D.

From the Wellcome Physiological Research Laboratories, Beckenham, Kent.

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In order to standardize anti-dysentery serum, it is necessary to have an accepted unit; before this can be established, a test toxin and test antitoxin must be available to all workers at the subject. In this paper we describe—

(1) Preparation and testing of "test toxin" (dried bacillary bodies); (2) Testing of "test anti-dysentery (Shiga) serum," as they have been carried out in these laboratories for some years past (Sudmersen and Eagleton, *Lancet*, 1921, **2**, 1109).

The animals employed were mice and rabbits; intravenous inoculation was used throughout.

Questions relating to different toxins, use of other animals and other methods, and many other obvious points of interest are not touched upon in this present paper.

Toxin.—"Toxin" is prepared by growing *B. dysenteriæ* (Shiga) on nutrient agar for 48 hours, washing off the growth in distilled water, heating just to the lethal point, *i.e.* 58° to 60 C. for 10 minutes, centrifuging and drying rapidly in a desiccator. The dried scales are finally ground so as to make a homogeneous powder.

For some reason which we cannot explain, some of the toxins prepared for a period of many months were poor in potency and irregular in action. The obvious causes of failure might be the use of improper "peptone," or of cultures of *B. dysenteriæ* (Shiga) which had become "rough." To neither of these causes could we definitely assign our occasional failures; we may remark in passing that "rough" colonies have produced as good toxin as "smooth" ones. We have for some months past been able to make supplies of antigen which have a satisfactory potency. Our first standard toxin, of which a supply is available, is called AA.

Results of intravenous injection into mice.—Animals of 20 gm. were used for all confirmatory tests, though frequently animals of 15 to 25 gm. were the only ones available for preliminary work. The dose, made up to 1·0 c.c. in sterile 0·75 per cent. solution of NaCl in distilled water, was injected into

* The substance of the present paper is to be presented as the first of a series of reports to the League of Nations Standardization Committee, which has requested workers in a number of different laboratories to investigate the subject.

one of the tail veins. The mice were inspected daily until the seventh day; those alive at this time were called survivors. In Table I are given the results of the injections made to ascertain the *minimum lethal dose.* Two to four mice were injected with each dose in each experiment. It will be seen that the range is fairly wide, a dose of 0·04 mgm. being needed to produce a fatal result in all mice, whereas some mice died when given 0·005 mgm.

TABLE I.—*Minimum Lethal Dose; Shiga Toxin AA.* (*Intravenous Injection: Mice.*)

Amount of toxin in mgm.	Number dying.	Number living.	% deaths.
0·1	1	0	100
0·05	1	0	100
0·04	6	0	100
0·03	5	1	83
0·025	13	3	81
0·02	13	3	81
0·015	8	6	57
0·01	9	9	50
0·0075	5	7	42
0·005	4	12	25
0·0025	0	8	0

The test dose of toxin.—A weighed amount of toxin is put into a stoppered cylinder containing the requisite volume of saline solution and shaken violently for a few moments to produce a homogeneous suspension; this is agitated between the withdrawal of each of the doses used in the experiment. The varying doses are then made up to 0·5 c.c. with saline solution and mixed with 0·5 c.c. of a saline solution containing 0·025 c.c. of our standard antitoxin, C.950, a total volume of 1 c.c. being injected. The mixture of toxin and antitoxin must stand for at least 30 minutes before injection.

Antitoxin, standard.—We have decided to adopt 0·025 c.c. of this serum C.950 as the standard test dose. This antitoxin of low potency was chosen because it was apparently stable. It has been in use for several years, and the amount, 0·025 c.c., was, as nearly as we could ascertain in tests continued for several years, the amount necessary to protect more than half the mice or rabbits injected with approximately 10 lethal doses of the original toxin which had been in use by one of us (H. J. S.) for some years. It will be seen from Tables I and II that if we take as the minimum lethal dose of toxin AA, 0·005 mgm., the lowest amount that kills an appreciable number of mice, then 0·025 c.c. of serum C.950 will protect an occasional mouse when injected with 25 to 30 minimum lethal doses, *i.e.* 0·125 mgm. or 0·15 mgm., and will protect more than 50 per cent. of those receiving 0·1 mgm. It is not easy, however, to name a certain quantity as the minimum lethal dose, and it is not therefore possible to say with accuracy against how many "minimum lethal doses" of the toxin AA the test dose of antitoxin will protect. For testing sera we decided to adopt for the present 0·2 mgm. of toxin AA as the test dose, since at that point any survival is apparently "significant." We

have in some of our work used 0·05 c.c. or 0·1 c.c. of this serum; it may be advisable to reconsider this point when dealing later with the "unit" of antitoxin for therapeutic purposes. Our results lead us to anticipate that if we double or quadruple the test dose of both toxin and antitoxin we shall obtain the same ratios for any series of antitoxins under test.

In Table III are summarized some series of experiments by two investigators working independently to ascertain the ratio between the dose of toxin

TABLE II.—*Determination of "Test Dose" of Toxin AA. (Intravenous Injection: Mice. Antitoxin C.*950, 0·025 *c.c. constant.)*

Amount of toxin in mgm.																	
0·25.		0·2.		0·15.		0·125.		0·1.		0·075.		0·05.		0·025.		0·015.	
3	.	3	.	3	.	3	.	3	.	3	.	2	.	4	.	s	
3	.	3	.	3	.	3	.	3	.	5	.	5	.	4	.	s	
2	.	5	.	4	.	3	.	3	.	5	.	7	.	s	.	s	
3	.	5	.	4	.	4	.	4	.	6	.	s	.	s	.	s	
4	.	3	.	5	.	4	.	4	.	6	.	s	.	s	.	s	
6	.	3	.	6	.	4	.	5	.	7	.	s	.	s	.	s	
4	.	3	.	3	.	4	.	7	.	s	.	s	.	s	.	s	
6	.	3	.	4	.	3	.	5	.	s	.	s	.	s	.	s	
4	.	3	.	5	.	4	.	s	.	s	.	s	.	s			
4	.	2	.	4	.	3	.	s	.	s	.	s	.	s			
4	.	4	.	4	.	7	.	s	.	s	.	s	.	s			
3	.	4	.	4	.	5	.	s	.	s	.	s	.	s			
4	.	3	.	4	.	7	.	s	.	s	.	s	.	s			
s	.	3	.	4	.	3	.	s	.	s							
				4	.	2	.	s									
				2	.	1	.	s									
				2	.	s	.	s									
				6	.	s	.	s									
				4				s									
				4													
				1													
				s													

Figures indicate day of death. s = survived 7 days.

TABLE III.—*Determination of "Test Dose" of Toxin AA. (Intravenous Injection: Mice. Antitoxin C.*950, 0·025 *c.c. constant.)*

Amount of toxin in mgm.	Number dying.		Number living.		% deaths 7th day.		% deaths 4th day.
	R.	S.	R.	S.	R.	S.	R.
0·25	22	—	0	—	100	—	100
0·2	35	14	0	0	100	100	88
0·15	32	21	3	1	93	95	68
0·125	15	16	5	2	75	84	55
0·1	11	8	9	11	54	44	20
0·05	3	3	5	10	37	23	37

R and S indicate two observers working independently.

which, when mixed with 0·025 c.c., our test dose of antitoxin, would cause 100 per cent. of deaths, and those causing approximately 20 per cent. of deaths.

The intervals between the doses are much wider than in standardization tests of diphtheria and anti-tetanic sera; the "error of the test" is of a different order. But with this reservation there is a satisfactory degree of agreement between the results obtained by two investigators working independently. In the final column are shown the percentages that would have resulted if the period of observation of the mice had been limited to four days, and all mice living after that time had been considered as survivors. We consider it preferable to adopt seven days as the limit of observation.

Results of intravenous injection into rabbits (Tables IV and V).—It was unfortunately impracticable during these experiments to obtain rabbits of a standard weight. It proved in practice to be more convenient to use whatever

TABLE IV.—*Minimum Lethal Dose Shiga Antigen AA. (Intravenous Injection: Rabbits.)*

Amount of toxin in mgm.	Number dying.	Number living.
0·05	2	0
0·025	2	1
0·015	0	2
0·01	0	2
0·005	0	1

TABLE V.—*Toxin (Shiga) AA against Antitoxin C.*950. *(Intravenous Injection: Rabbits. Antitoxin C.*950, 0·025 *c.c. constant.)*

Amount of toxin in mgm.	Number dying.		Number living.	
	R.	S.	R.	S.
0·2	2	—	0	—
0·175	1	—	1	—
0·15	5	8	4	1
0·1	3	9	6	2
0·075	0	1	3	5
0·05	—	0	—	4
0·025	—	0	—	1

R and S indicate two observers working independently.

rabbits were available and to give the desired dose of toxin without making any allowance for weight. We should have preferred to use rabbits of 1500 gm. for all experiments, but those that were available varied in weight from 1400 to 2500 gm. It is probable that our practice of making no allowance for weight is at least partly responsible for the small difference between the results of the two workers shown in Table V. It will be noted that one worker found that 0·15 mgm. of toxin AA when mixed with 0·025 c.c. of standard antitoxin and injected would kill 8 rabbits out of 9, whereas the other found only 5 out of 9.

When using a dose of 0·1 mgm. of toxin, one worker found that 9 out of 11 rabbits died, whereas the other found only 3 out of 9.

The dose chosen as a test dose of toxin is that which when mixed with 0·025 c.c. of C.950 would kill all rabbits injected; this dose is 0·2 mgm.

Summary.—A dysentery (Shiga) "toxin" consisting of dried bacillary bodies is described as a standard toxin for the titration of anti-dysentery sera.

The test dose of the toxin has been found by titration against a test dose of standard anti-dysentery (Shiga) serum.

We are at present undecided whether further experience will lead us to prefer mice or rabbits for standardization.

THE RELATION OF THE "SMOOTH" AND "ROUGH" FORMS OF INTESTINAL BACTERIA TO THE "O" AND "H" FORMS OF WEIL AND FELIX.

J. A. ARKWRIGHT AND AMAR NATH GOYLE.

From the Department of Bacteriology, Lister Institute, London.

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It has been shown that from most, if not all, strains of the "coli-typhoid-dysentery" group of bacteria two kinds of variants can be obtained, which have been called (1) "rough," distinguished from the normal by the fact that emulsions agglutinate in salt solution (NaCl 0·85 per cent.), and (2) "smooth," more closely resembling the normal and not agglutinated by salt solution. Evidence has also been brought forward that different "antigens" are characteristic of the two variants. The two antigens are distinct both as regards their corresponding agglutinins and the kind of clumping produced by specific sera (Arkwright, 1921). Breinl (1922) described spontaneously agglutinating variants of both Shiga's and Flexner's dysentery bacilli. He was not able in most cases to demonstrate receptors which were common to the variant and the parent strain, but his agglutination experiments were frustrated by his inability to secure stable emulsions. He does not, however, appear to have tried the effect of using solutions weaker than physiological saline.

It has also been shown by Weil and his fellow-workers that two forms which are characterized by other differences in their antigens occur as variants in the same group of organisms. This was first shown by Weil and Felix (1917) in the case of *B. proteus*, and later for *B. typhosus* and *B. paratyphosus* (Weil and Felix, 1918, 1920), for *B. typhosus* by Fürth (1922), for *B. enteritidis*, Gärtner, by Gruschka (1922).

These writers called the two forms, which they described, the "H" and the "O" form, because in the case of *B. proteus*, the first bacillus investigated, the "H" form was represented by the normal, which grew with a spreading film

(*Hauch*) on the surface of agar, whilst the "O" form, which occurred as a variant, grew in isolated colonies without any film (*ohne Hauch*). These two forms were further distinguished by their behaviour with agglutinating serum. An emulsion of the "O" form agglutinated in small granular clumps (*fein*), and was not altered in its agglutinability by being heated to 100° C. for one hour. The "H" form agglutinated in large loose flocculi (*grob*) which were dispersed readily by shaking, though some granular clumps were usually left behind. On heating an emulsion of the "H" form to 100° C. it was found that agglutination in large loose flocculi could no longer be obtained, but that some granular (*fein*) clumps formed with serum as in an emulsion of the "O" form.

The normal form was, therefore, described as containing two "receptors" or antigens, viz. the "H," which was heat-labile, and the "O," which was heat-stable, and the "O" form was considered to have only one antigen.

These conclusions were confirmed by absorption experiments and by making special sera with both heated and unheated emulsions of the two forms separately. Corresponding forms were found with the same relation between the two antigens in the case of *B. typhosus*, *B. paratyphosus*, *B. enteritidis* (Gärtner), but Weil and Felix (1920) state that *B. dysenteriæ* only possesses "O" receptors and that the "H" receptors are absent. Braun and Schaeffer (1919) found that though the "O" variants could often be isolated from old cultures of *B. proteus*, etc., their appearance could be hastened by growing cultures on agar containing 1 in 1000 carbolic acid, by using as a culture medium nutrient broth diluted ten times with water, or by certain other means which tended to repress or starve the bacteria.

Two sets of observations, then, have been made on these intestinal bacteria which show that strains can be split up into two forms. In the one case agglutination by salt has been taken as the criterion and the "S" (smooth) and "R" (rough) forms have been defined, of which the "S" appears to be nearest the normal and usually contains some of the "R" factor, whereas the "R" form often does not contain any of the "S" factor, as can be shown by its use for the preparation of serum, and for absorption of agglutinins from stock sera. In the other case the resistance to heat (100° C.) or the reverse has been used as the distinguishing feature of the two antigens. The normal has been called the "H" form, but contains both antigens "H" and "O," and the variant which has been isolated usually contains only the "O" antigen, which is heat-resistant.

THE OBJECT AND METHOD OF THE PRESENT WORK.

It was obviously desirable to determine whether the "S" and "R" forms corresponded in any way to the "O" and "H" forms, or whether they were due to entirely independent variation concerned with unrelated characters.

With this object observations were made on different organisms. One of us (A. N. G.) examined the variants of *B. typhosus* and *B. enteritidis* (Gärtner), the other (J. A. A.) those of *B. dysenteriæ* (Shiga).

For the purpose of selecting variants the "R" was distinguished from the "S" form by the agglutinability of an emulsion in 0·85 per cent. NaCl solution, and this form also was usually, but not always, recognizable by the formation of a deposit in broth cultures, leaving the medium clear.

Resistance to heat (100° C.) has been taken as the criterion for the "O" antigen and destruction by heat for the "H" antigen. The temperature used for this test has been obtained by plunging a test-tube containing the emulsion in a vessel of boiling water for 10 minutes in the case of *B. dysenteriæ* and 30 minutes for the emulsion of *B. typhosus* and *enteritidis*; by this means a temperature inside the tube of 99·5° C. was obtained. It was found that a temperature of 100° C. for half an hour as used by the continental workers was less satisfactory in some respects, since by this method emulsions of the "R" variants were often made much more easily agglutinable by salt solution, and sometimes clumped even in so weak a solution of NaCl as 0·21 per cent., but the less severe procedure adopted was usually found sufficient to completely abolish the agglutinability by serum of the heat-labile antigen, and had less effect in increasing the salt agglutinability.

It was noticed that if emulsions in salt solution of the "R" form were heated to 99·5° C. agglutination often occurred, which was so persistent that subsequently a good emulsion could not be obtained for use even in weak saline solution. For this reason, amongst others, the emulsions for agglutination were always made from agar cultures in distilled water containing 0·5 per cent. of carbolic acid, and the salt content raised at the time of the test to the desired level. In the case of serum agglutination the strength was, as a rule, 0·42 per cent., but if "spontaneous" agglutination occurred in this strength the content of sodium chloride was reduced to 0·21 per cent. or 0·1 per cent. The agglutination of heated and unheated emulsions of both smooth and rough forms of the same strain was always tested at the same time, and the same concentration of salt was used throughout each experiment. The "unheated" emulsions were not heated at all above 37° C.—the temperature of the incubator.

EXPERIMENTS WITH *B. TYPHOSUS* AND *B. ENTERITIDIS*.

The cultures for these experiments were provided by the National Collection of Type Cultures. Four strains of *B. typhosus*, Nos. 160, 162, 163 and 172, and one of *B. enteritidis*, No. 127, have been used.

The agglutinating sera were kindly provided by Dr. E. E. Atkin. The stock cultures when received were all "smooth" in character, *i. e.* they formed turbid cultures in broth and did not agglutinate in salt solution; they were regarded as belonging to the normal form. From all the five strains "rough" variants have been obtained by plating old broth cultures on agar and selecting colonies of irregular outline and coarsely granular surface. In order to obtain "smooth" variants containing as small a share of the "rough" factor as possible, the normal cultures were grown at 37° C. on nutrient agar to which 1 in 1000 carbolic acid had been added. On this medium daily subcultures were made. The growth was at first scanty, but after a few subcultures it became fairly abundant, but less so than on ordinary agar. The life of cultures on carbolic agar was not so long as on the ordinary medium. These "S" (smooth) variants have not been obtained by selecting colonies, and consequently may not be so pure nor so persistent as the "R" (rough) variants. The "S" cultures have, however, been shown to contain very little of the "R" component by the behaviour of emulsions, compared with those of the normal

form, in the presence of agglutinating serum before and after heating. The same conclusion has been arrived at from the results of experiments on the absorption of agglutinins, which will be recorded in another paper. Pure or nearly pure "S" forms were obtained from three strains of *B. typhosus*, Nos. 162, 163 and 172, and from the strain of *B. enteritidis*, No. 127.

Table I shows the agglutination results obtained with stock anti-typhoid serum and emulsions of normal, smooth and rough forms respectively, both before and after heating. Table II gives the corresponding results with the three forms of *B. enteritidis* and stock "Gärtner" serum.

The heated emulsions have been subjected to 99·5° C. for 30 minutes. Agglutination has been carried out in small test-tubes, which, after standing in the incubator at 37° C. for two hours, have been placed at the temperature of the laboratory for the night, and the final reading has been made in the morning.

In order to interpret the experiments of which the results are shown in Tables I and II, attention has been paid to three factors in the agglutination process:

(1) The character of the clumps.

(2) The end-point of the reaction, *i.e.* the highest dilution of serum which was effective.

(3) The rate at which the agglutination took place.

1. The clumps formed in emulsions of the "rough" form of *B. typhosus* and *B. enteritidis* are large, loose and flocculent-looking, like flakes of snow, as in the "*grob*" type of agglutination described by German writers.

The "smooth" form, on the other hand, agglutinates in the small compact granules (*fein*), which can usually be easily seen by the naked eye. These two types of agglutination are further distinguished by the fact that the "R" clumps disappear very readily on the tube being gently shaken; on the other hand the "S" granular agglutination is very little affected by moderate shaking.

Emulsions of normal strains form clumps of a mixed character; large loose flocculi appear, but disintegrate on being shaken and only some dense granules remain. These differences are most readily appreciated if the observation is made soon after removal of the tubes from the incubator, because on standing the large clumps settle down together and the flocculent character is to a great extent lost.

The granular clumps formed in agglutination of the "S" emulsion are sometimes so small they cannot be seen without a hand lens, especially at early stages of the agglutination and in tubes containing the weaker dilutions of serum. In the case of the "S" (granular) type of agglutination the deposit of clumps settles as a thin layer spread over the bottom of the tube, and frequently the edges of the deposit are folded over, giving it a star-shaped appearance, which is never seen in the case of the "R" agglutination. Whenever agglutination by serum of heated emulsions has occurred it has always been of the granular type.

2. The end-point of the agglutination with the normal and "R" emulsions has almost always been at the limit of the titre for the serum, with the exception of the normal form of *B. typhosus* 172. The "R" form of this

TABLE I.—*Agglutination of Normal, "S" and "R" Forms of* B. typhosus *with Stock Anti-typhoid Serum.*

Strain.	Form.	1/100.	1/200.	1/400.	1/800.	1/1600.	1/3200.	1/6400.	Control. NaCl 0·42 %.	Type of agglutination.	NaCl. M/2	NaCl. M/4	NaCl. M/8
160	Normal .	+++	+++	+++	+++	++	++	+	o	mx	o	o	o
	Heated do.	+++	+++	++	+	+	o	o	o	g	o	o	o
	R . .	++	++	++	++	++	+	+	o	fl	++	++	+
	Heated do.	+	+	o	o	o	o	o		g	++	++	+
162	Normal .	+++	+++	++	++	++	+	+	o	mx	o	o	o
	Heated do.	+	+	+	+	o	o	o	o	g	o	o	o
	S . .	+++	+++	+++	++	+	o	o	o	g	o	o	o
	Heated do.	+++	+++	++	+	sl	o	o	o	g	o	o	o
	R . .	+++	+++	++	++	++	+	+	o	fl	+++	+++	+
	Heated do.	+++	+++	+	o	o	o	o	o	g	++	++	+++
163	Normal .	++	++	++	+	+	+	+	o	mx	o	o	o
	Heated do.	+	+	+	sl	o	o	o	o	g	o	o	o
	S . .	+++	+++	+++	+++	+++	+++	+	o	g	o	o	o
	Heated do.	+++	+++	+++	++	++	++	+	o	g	o	o	o
	R . .	+++	+++	+++	++	+	+	+	o	fl	++	++	+
	Heated do.	+	+	o	o	o	o	o	o	g	+	+	++
172	Normal .	++	+	o	o	o	o	o	o	g	o	o	o
	Heated do.	+	+	+	sl	o	o	o	o	g	o	o	o
	S . .	+++	+++	++	+	+	o	o	o	g	o	o	o
	Heated do.	+++	+++	++	+	sl	o	o	o	g	o	o	o
	R . .	+++	+++	+++	++	++	++	+	o	fl	+++	+++	+
	Heated do.	+	+	sl						g	o	+	++

TABLE II.—*Agglutination of Normal, "S" and "R" Forms of* B. enteritidis (*Gärtner*) *with Stock Anti-Gärtner Serum.*

Strain.	Form.	1/100.	1/200.	1/400.	1/800.	1/1600.	1/3200.	1/6400.	1/12800.	1/25600.	Control. NaCl 0·42 %.	Type of agglutination.	NaCl. M/2	NaCl. M/4	NaCl. M/8
127	N . .	+++	+++	++	++	+	+	+	+	+	o	mx	o	o	o
	hN . .	+++	+++	+	o	o	o	o	o	o	o	g	o	o	o
	S . .	+++	+++	+++	++	++	+	o	o	o	o	g	o	o	o
	hS . .	+++	+++	++	+	o	o	o	o	o		g	o	o	o
	R . .	+++	+++	+++	+++	++	++	++	++	o	o	fl	+++	+++	+
	hR . .	sl	sl	o	o	o	o	o	o	o	o	g	+	+	+

NOTES ON TABLES I AND II.

The serum was diluted with NaCl 0·42 %.
N, S, and R = normal, "smooth" and "rough" forms.
h = heated emulsions; 99·5° C. for 30 minutes.
g = granular type of agglutination.
fl = flocculent " "

mx = mixed type of agglutination.
sl = slight agglutination.
NaCl $\frac{M}{2}$, $\frac{M}{4}$, $\frac{M}{8}$ = 2·9, 1·45, 0·725 %.

strain, however, reached a higher titre than either the normal or the "S" form. The "S" variants have not agglutinated as a rule in such high dilutions. Strain 163, "S" form, however, agglutinated to the full titre of the serum 1 in 6400, which may have been due to the fact that it had been grown longer than the other "S" forms on carbolic agar, and so contained a larger share of "S" antigen than the normal. This strain when grown on carbolic agar at first became less agglutinable, but on longer subculture it regained its agglutinability. The different effects of heat on the agglutinability of the three forms, normal, "S" and "R," is very clearly seen in Tables I and II. The "R" form after heating has seldom agglutinated in a higher dilution than 1 in 200, although the unheated emulsion usually reaches at least 1 in 6400.

The "S" form is scarcely affected by heating, whilst emulsions of the normal after heating may reach a considerably higher titre than before.

3. The rate of the reaction marks another decided difference between the agglutination of the different forms. In an unheated emulsion of the normal or "R" form loose flocculent clumps characteristic of the "R" antigen begin to appear early; they are seen in the highest or nearly highest dilution, *e.g.* 1 in 3200, immediately the tubes have been removed from the incubator after two hours at 37°C. In normal or "S" emulsions which have been heated or in unheated "S" emulsions, on the contrary, it is rare at this time for agglutination to be visible in any tube containing a higher dilution than 1 in 200, although after standing some hours agglutination up to 1 in 1600 may be quite evident.

The above experiments appear to demonstrate the existence of two antigens (agglutinable substances) in the normal form of *B. typhosus* and *B. enteritidis*. The "S" forms obtained correspond to the "O" forms of Weil and Felix and contain only heat-stable "O" antigen.

The "R" form which contains only the heat-labile antigen "H" was not isolated by Weil and Felix, or only rarely. In the normal form of these strains the "R" antigen is in evidence as regards agglutination by serum, because the "R" antigen appears to be responsible for the higher titre reached by the normal and "R" forms than by the "S" form.

EXPERIMENTS WITH *B. DYSENTERIÆ* (SHIGA).

Five strains of *B. dysenteriæ* (Shiga) have been used for these experiments, 550, 151, "H," "De" and "Be." For these we are indebted to the National Collection of Type Cultures.

Strain 550 had been "split" by one of us into "smooth" ("S") and "rough" ("R") forms in 1920 (Arkwright, 1921). The two forms were still available and had retained their distinctive characters after two years.

The other four strains were received in the "smooth" form and during the latter months of 1923 have been maintained in subculture, but in addition selected persistent "rough" variants have been obtained from them.

Several "S" and "R" variants have been isolated from each strain and these have varied considerably in their agglutinability, but the distinctive characters of the "S" and "R" form as regards agglutination in salt solution

NaCl $\frac{M}{2}$, $\frac{M}{4}$, $\frac{M}{8}$ = 2·9, 1·45, 0·725 %.
h = heated emulsions; 99·5° C. for 30 minutes.
g = granular type of agglutination.
fl = flocculent "

and agglutination with serum before and after heating have been the same in kind, though different in degree, for all the "S" and all the "R" forms respectively.

The forms of *B. dysenteriæ* labelled "S" are those selected cultures which appear to contain the least amount of the "R" element. They have not been obtained by growth on carbolic media.

TABLE III.—*Agglutination of Normal and "R" Strains of* B. dysenteriæ (*Shiga*) *with Stock-agglutinating "Anti-Shiga" Serum.*

Strain.	Form.	1/40.	1/80.	1/160.	1/320.	1/640.	Control. NaCl 0·2 %.	Type of agglutination.	NaCl M/4
550	S		+++	+	+	o	o	g	o
	hS		+++	+++	++	o	o	g	o
	R		+++	+++	sl	o	o	md	+++
	hR		o	o	o	o	o		+++
151	S	+++	+++	+++	sl	o	o	g	o
	hS	+++	+++	o	o	o	o	g	o
	R	+++	++	+	o	o	o	md	+++
	hR	+	o	o	o	o	o		+++
152	S	+++	++	o	o	o	o	g	o
	hS	+++	+++	sl	o	o	o	g	o
Ho	S	+++	+++	+	o	o	o	g	o
	hS	+++	+++	+++	++	o	o	g	o
	R	+++	+++	+	o	o	o	md	+++
	hR	o	o	o	o	o	o		+++
De	S	+++	+++	+	o	o	o	g	o
	hS	+++	+++	+++	o	o	o	g	o
	R	+++	++	o	o	o	o	md	+++
	hR	o	o	o	o	o	o		+++
							NaCl 0·1 %.		
Be	S		+++	+++	++	o	o	g	o
	hS		+++	+++	+++	sl	o	g	o
	R		+++	+++	+++	sl	o	md	+++
	hR		+	o	o	o	o		+++

The serum was diluted with NaCl 0·2 % for all strains except Be, when 0·1 % was used.
S = smooth. g = granular type of agglutination.
R = rough form. md = muddy " "
h = heated emulsion 99·5° C. for 10 minutes. sl = slight agglutination.

Table III shows the results of testing the "R" and "S" forms of different strains before and after heating to 99·5° C. for 10 minutes, with an agglutinating serum obtained with other strains of *B. dysenteriæ* (Shiga).

It is seen that the agglutination of the normal or of the "S" form is not impaired and is sometimes improved by previously heating the emulsion, and that the agglutination is granular in type.

The "S" form therefore contains the heat-stable "O" antigen as defined by Weil. The normal strains are culturally indistinguishable from the "S" form, since they are not agglutinated by salt solution and make a turbid growth in broth. They, however, contain some "R" antigen, since sera can be made from them containing agglutinins for the "R" form, which can be removed by absorption (Arkwright, 1921). "R" forms can probably always be obtained from the normal strains by plating out old cultures.

Emulsions of the "R" forms of *B. dysenteriæ* agglutinate in very small clumps, which settle so as to be spread out over the convexity at the bottom of the agglutination tubes. When these are shaken the contents become uniformly turbid to the naked eye (muddy type of agglutination). After heating, the agglutinability of the "R" form with serum is found to have been abolished. The "R" antigen, therefore, contains only the "H" or heat-labile antigen of Weil; the absence of loose flocculent (*grob*) clumps is a peculiarity of *B. dysenteriæ*, as opposed to *B. typhosus*, *proteus*, etc.

The fact that the heat-labile antigen in *B. dysenteriæ* does not form large flocculent clumps probably explains why Weil and Felix (1920) did not recognize the "H" antigen in dysentery bacilli.

The pure "R" form does not contain any "O" heat-resistant antigen.

GENERAL REMARKS.

The serological relations of the different forms described, "S," "R," "O" and "H," are therefore fairly clear.

The normal cultures have been shown to contain both "S" and "R" and also both "O" and "H" forms. The "S" antigen is heat-stable and is indistinguishable from the "O" antigen. The "R" antigen is heat-labile and identical with the "H" antigen. "S" has been taken as typical of the normal in this country because the normal cultures are "smooth," whereas on the continent the "H" antigen has been considered to be the mark of normal cultures, because they contain a heat-labile antigen. Unfortunately a different antigen has been made use of to name the normal form in the two countries whereas the "double form" would be more accurately descriptive. The reason of this is probably merely that the variant containing only one antigen which we have obtained pure most readily has been the "R" form, because as a criterion of variation we have taken agglutination by salt. Weil and his colleagues, on the other hand, first recognized a variant distinguished by having a single heat-stable antigen, "O." In the case of *B. typhosus* and *B. enteritidis* the variants produced by growth on carbolic agar have appeared to be pure "S" (="O") forms. There is little doubt that both kinds of pure variants containing respectively "S" (= "O") or "R" (="H") antigen alone can be obtained from most if not all strains of the coli-typhoid-dysentery group of micro-organisms if they are sought for. Weil and his colleagues and followers have made little mention of pure "H" (="R") variants in their work, but this has no doubt been because they have not looked for cultures which were agglutinable by salt, and have perhaps sometimes ignored them when found, on account of the difficulty in dealing with them for the purpose of agglutination experiments with specific serum, since these authors have apparently not used weaker solutions of saline than NaCl 0·85 per cent. even when they encountered unstable emulsions.

TYPES OF AGGLUTINATING SERUM.

The type of agglutination does not seem to be quite so constant a distinction between the two antigens as the effect of heat on emulsions. The latter is, therefore, on the whole the more valuable method of distinguishing the different forms.

Weil and Felix claimed that they could detect both types of agglutination in the same emulsion and this can sometimes no doubt be done readily, but with weak or much diluted sera the clumps in the granular, "O" (="S"), (*fein*) type may be very small and liable to confusion with the very minute clumps which occur in the other, "H" (="R"), especially after this has been dispersed by shaking. This difficulty is more liable to occur in the case of *B. dysenteriæ*, for then the "H" type of agglutination is only represented by "muddy" agglutination and large loose flocculi do not appear.

The different size and appearance of the clumps formed in emulsions of two varieties of the same bacillus was pointed out by Smith and Reagh (1903), who found that a non-motile strain of the hog-cholera bacillus agglutinated in fine granules, whereas the motile culture of what they believed to be the normal form of the same organism formed large loose clumps. They considered that the large clumps were due to the presence of flagella. They recognized that the motile variety excited the production of two kinds of agglutinin, one of which they considered corresponded to the flagella and the other to the bodies of the bacilli. These two Weil and Felix also recognized and attributed to the "H" and "O" antigens.

It has recently been again suggested that the antigen which flocculates in large clumps is derived from the flagella, and Braun and Schaeffer (1919), Feiler (1920), and Gruschka (1922), maintain that the heat-labile "H" antigen is directly related to the presence of flagella, since this type of agglutination does not occur with emulsions of the "O" form of *B. proteus*, *B. typhosus*, etc., which are non-motile. This hypothesis does not quite correspond with the facts, since the "R" (="H") form of *B. typhosus* is also often non-motile, and the heat-labile "H" antigen is characteristic of the "R" form of the non-motile *B. dysenteriæ*, though the clumps in this case are not loose and flocculent as in "R" forms of other species of bacteria described. It seems likely that the large loose "woolly" type of agglutination is associated with the large amount of bacterial substance contained in watery extracts of *B. typhosus* and *B. paratyphosus*, which readily gives a flocculent precipitate with agglutinating serum (Arkwright, 1914). *B. dysenteriæ*, on the other hand, gives up much less agglutinable substance to watery extracts. In the first case the clear extract probably does contain flagellar material, but the "R" form of *B. typhosus*, which is often non-motile, also yields an agglutinable watery extract. It is of interest that *either* of the two antigens present in a normal culture may be lost, and in this way a form with only one recognizable antigen may arise. Consequently if the single-antigen variants are to be regarded as degenerate forms, the deterioration has not always occurred along the same lines.

In several instances the agglutination by serum of the "S" form has definitely improved after heating the emulsion. It seems probable that this is due to some heat-labile "R" antigen being present in the emulsion, and exerting a "protective" action on the heat-stable antigen till after the application of heat.

EFFECT OF HEAT ON AGGLUTINATION BY SALT.

It was shown by Weil and Felix (1920) that the effect of 96 per cent. alcohol on emulsions of paratyphoid bacilli was similar to that produced by

heating to 100° C. In the course of the present work a few experiments have been made confirming this observation. "R" emulsions treated in this way have lost their agglutinability, whereas "S" emulsions have been unchanged.

An emulsion of a normal culture is not agglutinated by salt even though some "R" factor is present. This may be due to the "S" antigen "protecting" the "R" antigen.

Spontaneous agglutination of the "R" emulsion is not diminished by heating—in fact, it is usually increased, *i. e.* it occurs in lower concentrations of NaCl. This may perhaps be explained by the "R" factor being altered by heat so that the small amount of protection which it afforded to the bacterial bodies before heating is removed, and they are then more readily affected by the sodium ions.

CONCLUSIONS.

1. Normal strains of the *B. coli-typhosus-paratyphosus-dysenteriæ* group of intestinal organisms usually contain two kinds of antigen, "S" and "R," of which the latter is precipitated by salt solution (NaCl 0·85 per cent.) and the former is not.

2. By heating to 100° C. for ten minutes to one hour (or even to 99·5° C. for ten minutes) the "R" antigen is rendered inagglutinable by specific serum, whilst the "S" antigen is not affected. The "R" antigen therefore corresponds to the "H" receptor of Weil and Felix and the "S" to the "O" receptor.

3. The presence of the two antigens may be proved by demonstrating that the type of agglutination is changed after heating an emulsion, and that the heated emulsion will absorb the "S" (= "O") agglutinins but not the "H," and can be used for obtaining a pure agglutinating serum.

4. Variants can be obtained in which only one antigen is demonstrable. This remaining antigen may be either the "R" (= "H") or the "S" (= "O").

5. The form containing the "R" (= "H") antigen when agglutinated by serum is precipitated as a deposit, which is readily dispersed by shaking into a uniformly turbid emulsion, whereas the form with "S" (= "O") antigen agglutinates in granules which are not so readily broken up by shaking.

6. The "R" form has been obtained by selecting colonies from agar plates inoculated from old broth culture. The "S" forms of *B. typhosus* and *B. enteritidis* have been obtained by growing them on nutrient agar containing 1 in 1000 carbolic acid.

7. Emulsions of bacteria containing both antigens do not usually agglutinate in the presence of low concentrations of salt (*e. g.* NaCl 0·85 per cent.). Heating emulsions of the normal or "S" form does not make them more agglutinable by salt, but heating the "R" form as a rule renders it more readily agglutinable, *i. e.* in weaker salt solution.

8. In the case of *B. typhosus* and *B. enteritidis* (Gärtner), the "R" (= "H") type of agglutination with serum is in the form of large loose flocculi which are readily dispersed by shaking. Emulsions of the "R" form of *B. dysenteriæ* agglutinate in minute clumps which only form a small deposit. After being shaken, however, they become uniformly turbid (muddy type of agglutination), as also happens with the "R" form of *B. typhosus*,

etc. The agglutination of the "S" (= "O") form is similar for all these organisms and is of the granular type.

9. When specific serum agglutination is carried out with an emulsion in which both kinds of antigen are present (normal form), the agglutination is at first sight of the loose flocculent type in the case of the typhoid-paratyphoid-Gärtner sub-group; when the tubes are shaken a certain amount of turbidity is produced, but, in addition, granular clumps are usually seen in the resulting emulsion.

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SOME OBSERVATIONS ON THE PROPERTIES AND PREPARATION OF INSULIN, WITH SPECIAL REFERENCE TO THE PICRATE-ACETONE METHOD OF PREPARATION.

E. C. DODDS AND F. DICKENS.

From the Biochemical Department, the Bland-Sutton Institute of Pathology, Middlesex Hospital, London.

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THE history of insulin is so familiar that it is only necessary to make a brief reference to it here. The introduction of the substance by Banting and Best (1921) opened a new epoch in both diabetic therapy and chemical physiology; whilst the alcoholic extraction process worked out by Collip (1923) made insulin a practical remedy. It must always be remembered that the Toronto workers were the first to prepare insulin on a large scale from ordinary ox pancreas, and it is to them that we owe our knowledge of the method of fractional precipitation by means of alcohol.

As the properties of insulin became recognized, various other methods of preparation and purification were introduced. Thus purification by precipitation at the iso-electric point was used by Doisy, Somogyi and Shaffer (1923) in America; and Dudley (1923) in this country introduced his method of purification, based on the fact that insulin forms a picrate which is precipitated on the addition of picric acid to its aqueous solution. Shaffer and his collaborators have devised a method of preparation, of which the salting out of insulin from aqueous solution, by means of ammonium sulphate, provides the basis.

It seemed apparent that simpler methods of preparation could only be evolved from a wider knowledge of the chemical and physical properties of insulin, and it was decided to investigate the subject from this point of view.

The insulin used in the experiment to be described was prepared by Dudley's modification of the Collip alcohol process, and was subsequently purified by the former's picrate process. The rabbit unit after purification was 0·5 mg.

1. EXPERIMENTS ON THE SOLUBILITY OF INSULIN.

The experiments of Widmark (1923) were repeated with the pure hydrochloride and many of his observations were confirmed. The following solvents were tested and insulin was found to be freely soluble in them: (1) Glacial acetic acid; (2) phenol; (3) formamide. Paraldehyde was added to the list of solvents given by the above author.

It can be seen that the solubility of insulin is very similar to that of many proteins and protein derivations, and consequently little hope could be entertained of any method based upon the differential solubility of insulin and proteins in solvents other than alcohol.

2. THE SALTS OF INSULIN.

As the experiments on the solubility of insulin itself offered little encouragement, it was thought that perhaps the salts of insulin might differ in their behaviour from those of proteins.

Dudley was the first to recognize the importance of insulin picrate. This observer worked out the properties of this compound very fully, and based his well-known method of purification on his observations. In a recent paper (Dudley and Starling (1924)) the solubility of the picrate in wet alcohol is noted. We had observed this point independently, and Dr. Dudley informed us of his results prior to the publication of his paper.

The picrate was therefore the first salt investigated; it was prepared by adding a saturated solution of picric acid to an aqueous solution of insulin. The proportions recommended by Dudley were used, viz. 1·5 per cent. solution of insulin and half its volume of saturated picric acid solution. The picrate separates at once in the form of a pale yellow, flocculent precipitate, which soon settles to the bottom of the vessel. The supernatant fluid is then poured off and the picrate collected on a Buchner funnel, and washed with a dilute solution of picric acid. It is insoluble in water, in absolute alcohol, acetone or ether. It is easily soluble in wet acetone or alcohol, in formamide and in paraldehyde.

The solubility of insulin picrate in moist acetone is a very characteristic property. The optimum concentration of acetone was found to be about 70 per cent. Above and below this concentration the solubility decreases markedly. The acetone solution of the picrate is of a brownish colour and is perfectly clear; the picrate can be thrown out of solution by dilution with an equal volume of water and addition of saturated aqueous picric acid. The precipitate settles from this solution in the form of a light feathery mass. The precipitation is not complete, however, only about 60 per cent. being thrown out by this method. A quantitative separation can be obtained by removal of the acetone by distillation, either *in vacuo* or at ordinary pressure. In either case complete precipitation of the picrate is ensured by the addition of an equal volume of saturated aqueous picric acid to the residue. The picrate settles fairly rapidly, leaving the supernatant fluid clear enough for decantation in one or two hours. The temperature at which the distillation is carried out is immaterial as the following experiments show: One kg. of pig pancreas was extracted by the method described, and the acetone solution of the picrate so obtained was concentrated by distillation, at ordinary pressure, from a water-bath at 80° C. The precipitate of picrate, when worked up in the ordinary manner, gave a yield of 0·712 gm. of a perfectly white powder, the rabbit unit of which was 1 mg. The yield was therefore 712 rabbit units per kilo. A similar experiment under identical conditions, in which the distillation was conducted at 40° C. *in vacuo*, gave a yield of 675 rabbit units

per kilo, showing that there is no loss of activity due to the higher temperature of distillation.

It appears from the foregoing experiments that the picrate of insulin possesses very sharply defined solubility in mixtures of acetone and water, and that after distillation it can be completely precipitated from such a solution in an active form. The behaviour of protein picrates was next studied, in comparison with the properties of insulin picrate. For this purpose a series of picrates was prepared from blood, egg-albumin, and pancreatic proteins, by extracting these substances with water and adding picric acid in saturated solution. Picrates very similar to that of insulin were precipitated, and inactive hydrochlorides can be prepared from these picrates. Experiments on the solubility showed that the majority of protein picrates are insoluble in either absolute or aqueous acetone, insoluble in absolute alcohol, and sparingly soluble in aqueous alcohol. The solubility of insulin picrate is therefore very different from that of protein picrates; thus it was found that insulin could be separated from associated proteins by extraction of the mixture of picrates by means of aqueous acetone. The acetone extract contains the insulin picrate in solution; leaving the mass of protein picrates undissolved. This provided the basis of a simple method of extraction from the pancreas, which is described below.

A very peculiar point was encountered in some experiments with the alcoholic extract of the pancreas obtained by the Collip process. It will be remembered that the pancreas is extracted with an equal weight of 95 per cent. alcohol, filtered, and more alcohol added to bring the concentration up to 80 per cent. After standing overnight the clear supernatant liquid is decanted. This fluid contains insulin and proteins in solution, and it would be expected, therefore, to give a precipitate of protein picrates on the addition of an alcoholic solution of picric acid. It was very surprising to find that such a precipitate did not form, in spite of the fact that proteins and picric acid are present in the same solution, and that protein picrates are insoluble in moist alcohol. Acetone extracts of the pancreas behave in the same manner. It appears that when the proteins are actually in solution in moist alcohol or acetone it is impossible to precipitate them by means of picric acid, although the picrates themselves are insoluble in acetone or alcohol.

It was therefore impossible to devise any means of separation of the proteins and insulin, obtained by an alcohol extraction process, by means of picric acid in this way.

ACTION OF ORGANIC REAGENTS ON INSULIN.

All the solvents described up to the present are without action on the activity of insulin. Paraldehyde does not affect the activity. It was thought that the action of formalin might prove interesting from several points of view. In the first place the antiseptic action of formalin is undisputed, and it is perhaps the most powerful antitryptic agent known. If without effect on the activity, it would show either that there are no free NH_2 groups, or that these are not essential to the physiological action. The results of the experiments performed on this point are recorded below. Before injection into rabbits the formalin was removed by the action of ammonia.

Two series of experiments were performed, one at ordinary temperature the other at 37° C., using varying concentration of formalin. Below will be found a table expressing the results obtained. The insulin used was purified by Dudley's method and had a rabbit unit of 0·5 mg. Formalin of 40 per cent. concentration was added to the solution to bring the concentration of formaldehyde in the mixture to the percentage given. After the treatment set out in the table the solution was standardized in the usual way.

No.	% HCHO.	Temperature.	Time in hours.	Loss of activity.
1	20	Room	1 hour	*Nil.*
2	10	,,	1 ,,	,,
3	5	,,	1 ,,	,,
4	1	37° C.	½ ,,	,,
5	5	,,	½ ,,	Slight.
6	10	,,	½ ,,	Halved.
7	10	,,	1 ,,	Complete.

These experiments show that within the limits of error of the rabbit method of standardization there is no loss of activity with concentration of formaldehyde up to 20 per cent. at ordinary temperature, and little loss even at 37° C. with 5 per cent. formaldehyde solution.

With regard to the bearing of this observation on the constitution of insulin, it is difficult to express an opinion. Formalin attacks free NH_2 groups, and completely changes the character of proteins. Either there are no free NH_2 groups, which is extremely unlikely, or else these are not essential to the physiological activity, or possibly the compound with formalin, if formed at all, is very readily split by the action of ammonia.

In view of the fact that formalin is a powerful trypsin inhibitor, and is without action on insulin hydrochloride, except under sowewhat drastic conditions, it was thought that the addition of formalin either to the extracting fluid or to the pancreatic extract would increase the yield of insulin obtained by aqueous extraction. Pancreas was extracted with 0·1 per cent. formalin containing 1 per cent. of formic acid, but no active substance could be obtained, although a small yield was got when the formalin was omitted. A great variety of methods was tried, such as extraction with formic acid and addition of formalin to the expressed fluid, but all were without success. The explanation probably lies in the fact that the addition of formalin to a watery extract of pancreas produces a fine gelatinous precipitate which adsorbs the insulin. In addition, if formalin is present in the fluid used for the actual extraction, it will harden the tissues and thus greatly hinder the extraction.

METHODS OF PREPARATION OF INSULIN.

Armed with a knowledge of these properties of insulin, it is possible to devise new methods for the preparation of insulin without the use of the lengthy alcoholic extraction. That watery extracts of the pancreas could yield active material was known from the beginning of work on insulin, since Banting and Best's original method consisted in a water extraction of pancreas, and the first preparation of active substance was obtained in this manner.

When tried on a large scale, however, the yield was so poor as compared with the alcohol extraction process that the method had to be abandoned. Best and Scott state that out of some hundred experiments, their best yield obtained by aqueous extraction was only a quarter of that obtained by their alcohol process. Two causes of difficulty arise:

1. The inhibition of tryptic action.
2. Efficient separation of insulin from the relatively enormous mass of protein.

The first cause of loss was reduced, and the second difficulty completely overcome, by a method described by us in the *Lancet* of February 17th, 1924. Summarized briefly, the method consisted in extracting the pancreas with 1 per cent. formic acid, and precipitating the proteins and insulin from the resulting solution by means of picric acid. The picrates were then extracted with acetone. This separates the insulin picrate from the proteins, the acetone dissolving out the former and leaving the latter undissolved. The acetone solution was then diluted with equal volumes of saturated aqueous picric acid and water, when the insulin picrate separated. This was collected, washed and converted to the hydrochloride by Dudley's method.

The average yield by this method was in the neighbourhood of 300 rabbit-units per kilo of pancreas. This process was found to be difficult on a large scale owing to the time expended on the separation of the solid pancreatic tissue in the first stage. Also, in view of later experiments, it became obvious that about 40 per cent of the yield was lost by the incomplete precipitation of the picrate by dilution. In order to improve the process it was necessary—

1. To include some powerful tryptic inhibition to the extracting substance.
2. To adopt the removal of the acetone by distillation, before precipitating the picrate.

1. *Tryptic Inhibition.*

Many substances are known to inhibit the action of trypsin, but the majority also act upon insulin and impair its activity. Paraldehyde is free from this objection, and its addition was found to be advantageous.

The modified method differs from that described in the *Lancet* only in the addition of 5 per cent. of paraldehyde to the extracting fluid. Although paraldehyde is immiscible with water in this proportion, an emulsion is formed on mincing with the pancreas. The results of a typical experiment were as follow:

Experiment No. 10.—1 kg. of pig pancreas worked up by the above method gave 1·071 gm. of crude hydrochloride, the rabbit unit of which was 2 mg. The yield was therefore 535 rabbit units per kilo, as compared with 300 by the original process.

2. *Distillation.*

Some investigations on the properties of the picrate have shown the incompleteness of the precipitation from acetone solution obtained by the dilution method. It was thus clear that an increased yield should be obtained

as a result of distilling off the acetone. This procedure was adopted with good results, of which the following is a typical example.:

Experiment No. 9.—1 kg. pig pancreas was worked up by our first method. The acetone extract was concentrated by distillation under diminished pressure at 40°C. Weight of crude hydrochloride=0·269 gm. Rabbit unit =0·4 mg. Yield=675 rabbit units per kilo.

From these experiments it will be seen the yield can be greatly increased by either of these factors (1) and (2). The next step was to combine both of these modifications. This was done and the results were very successful, as the following experiment shows:

Experiment No. 19.—1 kg. pig pancreas was extracted as above and the acetone from the extract removed by distillation at 40°C. Yield of crude hydrochloride 1·040 gm. Rabbit unit=1 mg. The yield therefore is equivalent to 1040 rabbit units from 1 kilo of pancreas.

Although the results obtained by this process were consistently good, and the time factor was reduced to 2 days, there was still the objection that the first filtration was troublesome on a large scale, owing to the gelatinous nature of the material, although satisfactory yields could be obtained by this process.

In view of the ready solubility of insulin picrate in 70 per cent. acetone and the insolubility of protein picrates, it was an important point to ascertain whether this preliminary aqueous extraction of the pancreas was necessary, and whether picrates could not be prepared simply by grinding pancreas with solid picric acid. The application of picric acid to the original tissue, as a step in the extraction of insulin, was discussed in the course of a conversation with Dr. Dudley, who informed us that it was already under trial by him for fish islets.

One kg. of pancreas was minced and well mixed with 45·0 gm. of dry, powdered picric acid. After thorough mixing the whole was re-minced twice. A uniform pasty yellow mass was formed. This was extracted with 500 c.c. of dry acetone, and the clear liquid separated by filtration and pressure through "jean." The second and third extracts were made with 70 per cent. acetone. The separation of liquid is very complete and the solid material in the press should be quite dry. The acetone-picrate solution was then freed from acetone by distillation *in vacuo*, and the precipitation of the picrate rendered complete by the addition of 1 volume of saturated picric acid. The precipitate was collected and stirred with dry ether, whereby the fat and excess of picric acid is removed, leaving the pale yellow amorphous precipitate of picrate, which is then converted into the hydrochloride in the usual manner. The average yield of hydrochloride by this method was equivalent to about 1000 rabbit units from 1 kg. of pancreas, and had a rabbit unit of 0·5 to 1 mg.

We have had some batches showing even better results, the rabbit unit being as low as 0·25 mg. for the unpurified precipitate. In one experiment, Experiment 24, in which 8 kilos of pig pancreas were worked up by this method, we obtained our highest yield, namely 3·690 gm., the rabbit unit of which was 0·25 mg., thus giving a total yield of 14,760 rabbit units, or 1845 rabbit units per kg. of pancreas. So far as we know, this is the highest yield of insulin recorded.

After a series of experiments the following was adopted as the final method:

The pancreas is minced and well stirred with finely powdered picric acid (45 gm. per kilo of pancreas, which has been previously drained on a Buchner funnel). The mixture is again passed through the mincer to ensure even mixing, and it may be advantageous, particularly with frozen pancreas, to mince once more. Water usually separates during the mincing, and is pressed out in a hand-press through "jean" without difficulty. If frozen pancreas at a low temperature is used, however, little or no liquid separates, and this pressing may be omitted, a greater concentration of acetone being used in the first extraction (see below).

From the well-mixed picrated mass the picrate is then extracted with acetone. Three extractions are necessary, and the concentration of acetone in the extracting fluid should be 70 per cent. approximately. The quantity of acetone used in the first extract will vary with the amount of water present in the picric acid-pancreas mixture. We have obtained the best results by employing absolute acetone for the first extraction in sufficient quantity to make the final concentration to 70 per cent. In the succeeding extractions, aqueous acetone containing 70 per cent. of acetone should be used in quantity equal to half the weight of pancreas taken.

The method of extraction is as follows:

The minced pancreas-picric acid mixture is stirred with the requisite quantity of acetone, and efficient mixing is ensured by passing the whole mass through the mincer once again. This mixing should produce a paste of a creamy consistency, from which the liquid extract is pressed in a suitable press through a double layer of "jean." When no more liquid can be pressed out the solid material remaining is again minced into the required volume of 70 per cent. acetone and re-extracted as before. This process is repeated once more.

The combined extracts are filtered if necessary, and the filtrate distilled *in vacuo* until all the acetone is removed. From the aqueous residue after cooling a deposit of the amorphous precipitate of the picrate together with some fat and crystals of picric acid separate. It is as well to add an equal volume of saturated aqueous picric acid in order to ensure complete precipitation. If the precipitate contains a large amount of picric acid crystals the addition of the saturated picric acid solution is, of course, superfluous. The precipitate is collected on a Buchner funnel, washed by stirring with ether, and the ether filtered off. The picrate remains undissolved, whilst the fat and excess of picric acid are removed by washing with ether on a Buchner funnel. So obtained, the picrate is a pale yellow, amorphous powder, which is readily converted into the hydrochloride by Dudley's method.

For this purpose it is dissolved in acid alcohol prepared by mixing 25 c.c. of aqueous 3N HCl with 75 c.c. of alcohol. Ten to twenty c.c. of this mixture are usually required for each gramme of picrate. By careful rubbing with a glass rod, a turbid solution of a dark brown colour is obtained. The solution is then centrifuged, and the supernatant fluid is poured off. The solid material in the bottom of the tube is ground up with a further quantity of the acid alcohol solution, and is centrifuged again. It is as well though not absolutely

necessary to extract a third time. From the resulting clear fluid the hydrochloride is precipitated by the addition of ten to twenty volumes of acetone. The hydrochloride is allowed to settle, and the clear supernatant fluid is decanted. The remainder is poured on to a Buchner funnel, and the precipitate is washed with acetone until free from picric acid, and finally with dry ether. It is then dried in a vacuum desiccator. The crude hydrochloride so obtained is a perfectly white, non-hygroscopic, amorphous powder, the rabbit unit of which lies between 0·25 to 1 mg.

Comments on the Method.

1. The use of solid picric acid not only renders the first tedious filtration unnecessary, but by converting the insulin directly to picrate, reduces the time during which tryptic action may occur to a minimum, thus increasing the yield.

2. By centrifuging the acid-alcohol solution of the picrate, in the last stage, a large quantity of insoluble material is removed. This consists of protein insoluble in 75 per cent. alcohol, and is quite inactive physiologically. It probably consists of part of the fraction which is precipitated at an earlier stage in the Collip process. The removal of protein in this way gives a product of a very high degree of purity.

3. The rabbit unit of the "crude hydrochloride" is in the neighbourhood of 0·5 mg., which is as low as that of the purified insulin made by the Collip process and subsequently purified by Dudley's method.

4. The time of the complete process is about one day. In this laboratory we have worked up small batches of up to 10 kilos within 6 hours.

5. The process presents no difficulties with regard to filtration, as all the precipitates are very easily handled.

The rabbit units given in this paper are in terms of the original Toronto units, viz. the quantity required to lower the blood-sugar of a 2-kilo rabbit to 0·045 per cent. within 2 hours. In order to conserve space the protocols of the standardization have been omitted throughout.

In conclusion we wish to record our appreciation of the interest shown by Dr. Dale and Dr. Dudley, of the Scientific Staff of the Medical Research Council, and to thank them for many helpful suggestions. We have to express our thanks to Prof. McIntosh, Director of the Bland-Sutton Institute of Pathology, for his interest and advice throughout the whole of this work.

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